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Estudo genético da doença de Parkinson

Tese apresentada à Faculdade de Medicina
da Universidade de São Paulo para
obtenção do título de Doutor em Ciências

Área de concentração: Neurologia

Orientador: Prof. Dr. Egberto Reis Barbosa

São Paulo

2007

Dados Internacionais de Catalogação na Publicação (CIP)

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Chien, Hsin Fen

Estudo genético da doença de Parkinson / Chien Hsin Fen. -- São Paulo, 2007.

Tese(doutorado)--Faculdade de Medicina da Universidade de São Paulo.
Departamento de Neurologia.

Área de concentração: Neurologia.

Orientador: Egberto Reis Barbosa.

Descritores: 1.Doença de Parkinson/genética 2.Transtornos
Parkinsonianos/genética 3.Mapeamento cromossômico 4.Fenótipo
5.Hereditariedade

USP/FM/SBD-009/07

“Porque de Deus, e por meio Dele, e para Ele são todas as coisas.

A Ele, pois, a glória eternamente. Amém.”

Romanos 11:36

Aos meus pais

“Com amor eterno eu vos amei.”

Jeremias 31:3

Ao Prof. Dr. Egberto Reis Barbosa

“O ensino do sábio é fonte de vida.”

Provérbios 13:14

Ao Prof. Dr. Vincenzo Bonifati

“A alma generosa prosperará.”

Provérbios 11:25

Ao Ioannis

*“O amor é paciente, é benigno...tudo sofre, tudo crê, tudo espera, tudo
suporta.”*

I Coríntios 13: 4a e 7

AGRADECIMENTOS

Ao meu irmão Marcos, minha cunhada Gleice e ao David.

Aos meus tios Minteng e Susie Tahn.

À ministra e amiga Chen Li Hwei.

Ao Sr. Michel e D^a. Eftimia, Afroditi e Jorge.

Ao Dr. Wu Tu Hsing.

À Dra. Maria do Desterro Leiros Costa.

Ao Prof. Dr. Manoel Jacobsen Teixeira.

Ao Prof. Dr. Fernando Kok.

À Dra. Patrícia Aguiar.

Ao Dr. João Carlos Aparecido Pereira.

À Dra. Mariana Spitz.

Ao Dr. Flávio A. Sekeff Sallem.

Ao Dr. Roberto Rozenberg.

Aos membros do LIM-25 da FMUSP, Dra. Sandra Maria Ferreira Villares, Eliana Salgado Turri Frazzatto e Isabel Cristina de Mello Guazzelli.

Ao Departamento de Genética Clínica do Centro Médico da de Universidade Erasmus, Rotterdam.

A todos os amigos, colegas e funcionários do Departamento de Neurologia do Hospital das Clínicas da FMUSP.

Aos meus pacientes e seus familiares, sem os quais esse trabalho não teria sentido.

**Esta tese de doutorado foi desenvolvida com bolsas de estudo
concedidos pelo Conselho Nacional de Desenvolvimento Científico
(CNPq) e pela Coordenação de Aperfeiçoamento de Pessoal de Nível
Superior (CAPES)**

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LISTA DE SIGLAS

AD	Autossômico dominante
AR	Autossômico recessivo
BH4	Tetrahidrobiopterina
CL	Corpúsculo de Lewy
DNA	Ácido desoxirribonucleico
DNAc	Ácido desoxirribonucleico complementar
dNTP	Desoxirribonucleotídeo trifosfatado
DP	Doença de Parkinson
H&Y	Escala de Hoehn and Yahr
HUGO	Organização do Genoma Humano
LRRK2	Quinase rica em repetição de leucina 2
RNA	Ácido ribonucleico
RNA _m	Ácido ribonucleico mensageiro
ROS	Espécies reativas de oxigênio
RT-PCR	Transcrição reversa - Reação polimerásica em cadeia
SNCA	Alfa-sinucleína
SPR	Sepiapterina redutase
PCR	Reação polimerásica em cadeia
PINK1	PTEN-induzida quinase 1
PP	Parkinsonismo primário
UCHL1	Ubiquitina carboxi terminal esterase L1
UPDRS	Escala unificada de avaliação da doença de Parkinson

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RESUMO

Chien HF. *Estudo genético da doença de Parkinson* [tese]. São Paulo: Faculdade de Medicina, Universidade de São Paulo; 2006. 106p.

A doença de Parkinson (DP) é a segunda doença neurodegenerativa mais comum com uma prevalência aproximada de 3% em pacientes com mais de 64 anos. A doença é esporádica, mas o parkinsonismo primário (PP) familiar, decorrente de defeitos genéticos específicos, tem sido encontrado em cerca de 10% dos casos diagnosticados como DP. Os objetivos deste trabalho são analisar o DNA de pacientes com PP acompanhados no ambulatório do Grupo de Estudo de Distúrbios do Movimento da Clínica Neurológica do Hospital das Clínicas da FMUSP que apresentam início precoce (< 40 anos) ou história familiar positiva com o intuito de rastrear mutações responsáveis pela doença e descrever as características clínicas desse grupo de pacientes e dos familiares acometidos. Entre Janeiro de 2004 a Janeiro de 2006 foram selecionados 53 probandos com PP, sendo que 29 eram esporádicos, 16 com história familiar sugestiva de herança autossômica dominante (AD) e 8 com história familiar sugestiva de herança de autossômica recessiva (AR). No total, 100 amostras de DNA foram coletadas, 70 de pacientes ou familiares com PP, 1 com parkinsonismo secundário ao uso de neuroléptico e o restante de familiares sem PP. Dos casos afetados, 45 eram do sexo masculino e 25 feminino, a idade média de início dos sintomas foi de 38,3 anos (10-72) e a média de idade no momento da investigação foi de 49,8 anos (22-72). Todos apresentaram instalação assimétrica do quadro, curso lento e progressivo e boa resposta ao tratamento com levodopa ou agonista dopaminérgico. Pacientes com padrão de herança AD foram testados para a mutação Glí2019Ser que é o defeito mais comum do gene *LRRK2* (*PARK8*) sendo encontradas duas famílias afetadas. A análise mutacional dos genes *PARK6* e *PARK7* está em andamento. Todos os casos esporádicos e com padrão de transmissão AR foram testados para mutações do gene *PARK2* e foram encontradas as seguintes mutações homozigóticas em 4 famílias: 255delA, deleção de exon 3-4, deleção do exon 2-3 e uma nova mutação IVS1+1G/T. Num paciente com parkinsonismo juvenil (idade de início dos sintomas <21 anos) foi encontrada uma nova mutação homozigótica no gene *ATP13A2* (*PARK9*) no exon 15 que determina a substituição Glí504Arg na proteína codificada. Em grande parte dos casos estudados os achados genéticos e clínicos são similares aos descritos na literatura. Entretanto, encontramos novas mutações do gene *PARK2* e *PARK9* e no paciente com a mutação *ATP13A2* os achados clínicos diferem em alguns aspectos da descrição clássica.

Descritores: 1. Doença de Parkinson/genética; 2. Transtornos parkinsonianos/ genética; 3. Mapeamento cromossômico; 4. Fenótipo; 5. Hereditariedade.

SUMMARY

Chien HF. *Genetical study of Parkinson's disease* [tese]. São Paulo: "Faculdade de Medicina, Universidade de São Paulo"; 2006. 106p.

Parkinson disease (PD) is the second most common neurodegenerative disorder affecting approximately 3% of the population over age 64. Most cases of PD manifest in sporadic form, but familial primary parkinsonism (PP) due to specific genetical abnormalities has been found in about 10% of cases diagnosed as PD. The aims of this study were to analyze the DNA of PP patients seen at the Group for the Study of Movement Disorders of the Neurology Department of Hospital das Clinicas of the University of São Paulo who presented early onset of the disease (< 40 years of age) or positive family history, with the purpose of screening possible candidate mutations for the disease, and to describe the clinical features of this group of patients and affected members of their families. Between January 2004 and January 2006, 53 probands were selected of whom, 29 were sporadic cases, 16 had probable autosomal dominant (AD) pattern of inheritance, and 8 autosomal recessive (AR). In total 100 samples of DNA were collected, 70 from PP patients or affected relatives, one case with neuroleptic-induced parkinsonism, and the rest from not affected members. Forty five affected individuals were men and 25 women, the median age of the symptoms onset was 38.3 years (10-72), and the median age at the moment of the examination was 49.8 years (22-72). All patients had asymmetric installation of the disease, slow progression of the PP, and good response to levodopa or dopaminergic agonist therapy. Patients with AD inheritance were screened for Gly2019Ser mutation, which is the most common defect in PD due to *LRRK2* gene, and two families carried this mutation. The screening of *PARK6* and *PARK7* is ongoing. All sporadic and AR inheritance cases were tested for mutation of (*PARK2*) and the following mutation were found in 4 families in homozygous state: 255delA, exon 3-4 deletion, exon 2-3 deletion, and a novel mutation IVS1+1G/T. In a juvenile parkinsonism proband (age of onset < 21 years) a novel missense homozygous mutation in *ATP13A2* (*PARK9*) gene was found in exon 15 which resulted the Gly504Arg change in the encoded protein. In general the genetical and clinical findings of this series of patients are similar to those reported in the literature, although novel mutation in *PARK2* and *PARK9* were obtained. Some clinical features of the patient with *ATP13A2* mutation differed from the classical descriptions.

Keywords: 1. Parkinson disease/ genetics; 2. Parkinsonian disorders/ genetics; 3. Chromosome mapping; 4. Phenotype; 5. Heredity.

INTRODUÇÃO

INTRODUÇÃO

1.1 Prefácio

Durante o 5º Congresso Internacional de Doença de Parkinson e Distúrbios do Movimento, organizado pela *The Movement Disorder Society*, realizado em Nova Iorque em 1998, o Dr. Egberto Reis Barbosa foi apresentado ao Dr. Vincenzo Bonifati, na ocasião um desconhecido, mas promissor jovem neurologista que se dedicava ao estudo da genética em doença de Parkinson.

Ciente do potencial genético nos estudos da doença de Parkinson e da dedicação do jovem cientista, Dr. Egberto estabeleceu uma relação acadêmica com Dr. Bonifati e em maio de 2002, convidando-o para participar como palestrante principal do II Simpósio Paulista sobre Distúrbio do Movimento da Associação Paulista de Medicina sobre novos avanços na genética da doença de Parkinson. Na ocasião também foi estabelecida uma parceria entre ambos para pesquisa sobre o assunto.

Estimulada, pelo Dr. Egberto iniciei a minha participação nesse projeto em 2003. O ponto de partida na seleção de casos foi o paciente (PDBR01.96) que iniciara os sintomas parkinsonianos com a idade de 14 anos e que estava em acompanhamento no Ambulatório do Grupo de Estudo de Distúrbios do Movimento há mais de 20 anos. Posteriormente, outros familiares acometidos vieram da Paraíba para serem acompanhados

no serviço e percebeu-se que muitos eram acometidos porque havia nesta família a prática de casamentos consanguíneos há varias gerações.

Viajei para o interior de Paraíba em setembro de 2003 para estudar a família. A experiência foi marcante e voltei para São Paulo determinada a aprofundar os meus conhecimentos na área de genética na DP. Esta viagem posteriormente resultou na publicação de um artigo sobre essa família na revista *Neurogenetics* (Chien et al., 2006).

1.2 Doença de Parkinson

A doença de Parkinson (DP) é a segunda doença neurodegenerativa mais comum (Lang e Lozano, 1998), com uma prevalência de 3,3 % acima dos 64 anos de idade (Barbosa et al., 2006).

O diagnóstico é clínico e baseia-se na presença dos sinais cardinais: tremor, rigidez, bradicinesia e instabilidade postural. (Barbosa et al., 1997). Outros achados, como excelente resposta ao tratamento com levodopa, início unilateral e persistência da assimetria do quadro auxiliam no diagnóstico (Hughes et al., 1992).

Para se estabelecer o diagnóstico definitivo da DP idiopática é necessário a confirmação da presença de corpúsculos de Lewy (CL) na substância negra no estudo anátomo-patológico (Hughes et al., 1992).

O CL é uma estrutura esférica de 8 a 30 μ m de diâmetro, de inclusão intracitoplasmática, de coloração rósea quando corado com a hematoxilina e

eosina. Os CL encontrados na substância negra tipicamente têm o centro intensamente corado e a periferia com um halo levemente corado enquanto que os CL dos neurônios corticais têm um aspecto mais homogêneo sem o contraste de centro e halo periférico. O centro do corpúsculo contém material granular denso e a região periférica filamentos com arranjo radiado (Olanow et al., 2004).

As primeiras descrições de história familiar de DP surgiram no final do século XIX. Gowers verificou que 15% dos seus pacientes com DP apresentavam história familiar positiva (Gowers, 1893 apud Polymeropoulos et al., 1996). Estudos epidemiológicos têm explorado a frequência do parkinsonismo primário (PP) familiar e as pesquisas com gêmeos monozigóticos e dizigóticos foram os meios de investigação iniciais para distinguir a contribuição genética e os riscos do meio ambiente para a manifestação da DP (Ward et al., 1983; Burn et al., 1992; Foltynie et al., 2002). A análise do genoma de famílias acometidas visa pesquisar e identificar novos genes envolvidos na transmissão da doença. A tabela 1.1 mostra os *loci* genéticos que estão envolvidos na manifestação de parkinsonismo.

Tabela 1.1 : Genes envolvidos no parkinsonismo familiar

Nome do Locus	Gene	Locus	Proteína	Herança	Referência
PARK1	<i>SNCA</i>	4q21-q23	“-synuclein” (-sinucleína)	AD	Polymeropoulos et al. 1997
PARK2	<i>PARK2</i>	6q25-q27	“parkin” (parkina)	AR	Kitada et al. 1998
PARK3	—	2p13	—	AD	Gasser et al. 1998
PARK5	<i>UCHL1</i>	4p14	“ubiquitin C-terminal esterase L1” (UCHL1)	AD	Leroy et al. 1998
PARK6	<i>PARK6</i>	1p35-p36	“PTEN-induced kinase1” (PINK1)	AR	Valente et al. 2004
PARK7	<i>PARK7</i>	1p37	DJ-1	AR	Bonifati et al. 2003
PARK8	<i>LRRK2</i>	12q12	“leucine-rich repeat kinase 2” (LRRK2, dardarina)	AD	Paisan-Ruiz et al. 2004; Zimprich et al. 2004
PARK9	<i>ATP13A2</i>	1p36	ATP13A2	AR	Ramirez et al. 2006
PARK10	—	1p32	—	AD?	
PARK11	—	2q34	—	AD?	

AD= autossômico dominante; AR= autossômico recessivo

Sete genes com padrão de transmissão Mendeliano foram identificados até o presente momento. De acordo com a nomenclatura adotada pela HUGO (*Human Genome Organisation*) de 01 de dezembro de 2006, os genes das formas autossômicas dominantes (AD) são: *SNCA*, *UCHL1* e *LRRK2*. As recessivas (AR) são *PARK2*, *PARK6*, *PARK7* e *ATP13A2*.

Quanto aos *PARK10* e *PARK11* estes são *loci* de susceptibilidade e não têm um modo definido de transmissão (Pankratz et al., 2003).

Acredita-se que apenas 10 a 15% dos casos de parkinsonismo primário familiar sejam monogênicos (Bonifati et al., 2004a). Dessa forma, os fatores poligênicos e ambientais ainda são preponderantes na etiologia do PP (Figura 1.1).

Figura 1.1: Padrão de herança em DP

PD: doença de Parkinson; AR: herança autossômica recessiva; AD: herança autossômica dominante. Extraído de Bonifati et al., 2004a.

Na investigação genética além da história familiar, manifestação clínica, curso da doença, consangüinidade e etnia do paciente, a idade de início dos sintomas é importante. As formas monogênicas de PP podem ser de origem esporádica ou familiar e geralmente manifestam-se mais precocemente que os casos de DP. Quando a doença inicia-se antes dos 20 anos de idade é denominada de parkinsonismo juvenil, e entre os 20 aos 40 anos de parkinsonismo de início precoce (Paviour et al., 2004). Geralmente os aspectos clínicos do parkinsonismo genético são indistinguíveis da DP.

Dentre as formas familiares a mutação do gene *PARK2* é o mais freqüentemente alterado, ao passo que nas formas esporádicas é o gene *LRKK2*. A tabela 1.2 a seguir mostra a freqüência de mutação dos genes conhecidos até o presente momento encontrados nos parkinsonismos monogênicos.

Tabela 1.2: Frequência estimada dos genes nas formas familiares e esporádicas

Forma familiar		Esporádica	
<i>PARK2</i>	10-20%	<i>LRRK2</i>	2%
<i>PARK6</i>	2-7%		
<i>PARK7</i>	1-2%	<i>PARK2</i>	
<i>LRRK2</i>	5-10%	<i>PINK1</i>	Raros
<i>SNCA</i>	<0,5%	<i>PARK7</i>	

Tabela adaptada de Klein e Schlossmacher (2006b).

Contudo, se considerarmos rigorosamente os critérios estabelecidos pelo Banco de Cérebro de Londres para o diagnóstico de DP (Hughes et al., 1992) um dos critérios de exclusão é a positividade de história familiar para a doença. Dessa forma, nenhum dos casos de parkinsonismo por causa genética deveria ser considerado DP, apesar de muitas vezes as características clínicas do parkinsonismo genético serem indistinguíveis da DP idiopática.(Hardy et al., 2006). A questão da classificação e denominação das síndromes parkinsonianas é importante, mas para este estudo denominamos as formas genéticas de parkinsonismo e restringimos o termo doença de Parkinson para o quadro clássico, idiopático que preenchem os critérios do Banco de Cérebro de Londres (Hughes et al., 1992).

Mantivemos no título o termo Doença de Parkinson porque na literatura os casos genéticos de parkinsonismo ainda são denominados de DP.

1.3 Objetivo

Os objetivos do presente estudo são:

1. Investigar a ocorrência de mutações de genes responsáveis pelo parkinsonismo de pacientes e familiares acompanhados no Ambulatório do Grupo de Estudo de Distúrbios do Movimento da Clínica Neurológica do Hospital das Clínicas da FMUSP
2. Descrever as características clínicas dos indivíduos nas quais mutações forem encontradas.

REVISÃO DA LITERATURA

REVISÃO DA LITERATURA

2.1 Parkinsonismo de transmissão autossômica dominante

SNCA

Em 1996, Polymeropoulos et al. demonstraram que numa grande família ítalo-americana originária de Contursi (Itália) com PP e padrão de transmissão AD (Golbe et al., 1996) havia segregação da doença com marcadores em 4q21-23. O *locus* foi denominado PARK1.

Um ano após, o mesmo grupo (Polymeropoulos et al., 1997) identificou uma mutação de ponto, cG209A, Ala53Tre, no gene *SNCA* que codifica a proteína α -sinucleína na família de Contursi e outras três famílias gregas. A análise de haplótipo sugere haver um ancestral comum entre essas famílias, explicando a presença da mesma mutação (Athanassiadou et al., 1999).

Uma segunda mutação no gene *SNCA*, G88C, que gera uma substituição Pro30Ala na proteína α -sinucleína, foi encontrada em uma família de origem germânica (Kruger et al., 1998). Recentemente uma terceira mutação, G188A (Glu46Lis), foi identificada numa família espanhola (Zarranz et al., 2004). Entretanto, nesta última família, os fenótipos variavam entre a forma clássica da DP e demência com corpúsculos de Lewy.

Vários pesquisadores em diversos países, inclusive no Brasil, tentaram identificar mutações no gene *SNCA* em casos esporádicos ou familiares de DP, mas os resultados indicam que são muito raras (Chan et al., 1998;

Farrer et al., 1988; Ho e Kung, 1998; Vaughan et al., 1998; Teive et al., 2001).

A presença de múltiplas cópias do gene *SNCA* foi primeiramente detectada em uma família de Iowa (EUA) que apresentava uma triplicação do gene e o dobro da concentração de α -sinucleína no sangue periférico (Singleton et al. 2003; Miller et al., 2004). Essa família fora anteriormente classificada erroneamente como uma nova forma de parkinsonismo e o gene denominado de *PARK4* (Farrer et al., 1999). Posteriormente outras famílias em que indivíduos apresentavam duplicação do gene também foram descritas (Chartier-Harlin et al., 2004; Ibanez et al. 2004).

A importância da descoberta da mutação ou multiplicação do gene *SNCA* consiste no fato de que a proteína α -sinucleína é um dos principais componentes do CL, marcador da DP (Spillantini et al., 1997) e de outras α -sinucleinopatias.

O quadro clínico dos pacientes com mutação do gene *SNCA* diverge dos casos de DP clássica pela precocidade das manifestações clínicas, em torno dos 40 anos, e rápida progressão da doença (Golbe et al., 1996). Nota-se também menos tremor e maior predominância do quadro rígido-acinético. Além disso, os pacientes podem apresentar demência (Bostantjopoulou et al., 2001), disfunção autonômica (Papapetropoulos et al., 2001 e 2003), hipotensão postural, mioclonia e hipoventilação central (Spira et al. 2001). Similarmente aos casos idiopáticos, os pacientes respondem bem à levodopa e exibem os efeitos colaterais típicos da droga (Golbe et al., 2001; Papapetropoulos et al., 2001 e 2003).

Nas famílias com triplicação do gene o início do parkinsonismo é precoce, o curso da doença é rápido e o quadro clínico variável. Por outro lado, famílias com duplicação do *SNCA* apesar do início precoce em relação aos casos de DP apresentam manifestação clínica mais tardia e o curso mais prolongado que nas famílias com triplicação do gene. Observa-se também maior incidência de demência nessas formas da doença. Portanto, esses dados sugerem que o fenótipo da doença nessas famílias está diretamente relacionado com o aumento da expressão da α -sinucleína selvagem (Nishioka et al., 2006).

Os achados anátomo-patológicos na família de Contursi, assim como na de Iowa evidenciavam presença difusa de CL (Bonifati et al., 2004a).

PARK3

O *locus* PARK3, localizado no cromossomo 2 (2p13), foi mapeado em seis famílias com padrão de transmissão autossômico dominante e descrito por Gasser et al., em 1998. O padrão clínico não difere dos casos de DP apesar do relato de incidência de demência em duas famílias. A idade de manifestação variou de 37 a 89 anos e o estudo anátomo-patológico demonstrou a presença de CL (Dekker et al., 2003).

Várias evidências sugerem que o gene *SPR* (*Sepiapterin Reductase*) que codifica a proteína sepiapterina redutase é um forte candidato para *PARK3*. Uma delas é que em estudos com múltiplas famílias, o marcador microssatélite D2S1394, que está a 9 Kb do gene *SPR* tem influência na

idade de início da DP. Outros marcadores ao redor ou ao longo do gene *SPR* também foram correlacionados com DP (Sharma et al., 2006).

Recentemente, Steinberger et al. (2004) relataram um caso com distonia levodopa responsiva que apresentava uma mutação na região 5' não traduzida do gene *SPR*. A paciente era heterozigota e por ser adotada, não foi possível investigar a família de origem. A sepiapterina redutase catalisa a conversão de 6-piruvoil-tetrahydropterina em tetrahydrobiopterina (BH4). A BH4 é cofator da tirosina hidroxilase que converte a tirosina em levodopa.

UCHL1 (PARK5)

Uma mutação no gene que codifica a proteína UCHL-1 (*ubiquitin carboxy-terminal esterase 1*) foi identificada em dois membros de uma família alemã com parkinsonismo de transmissão AD. Esse gene foi denominado de *UCHL1* (previamente denominado *PARK5*) (Leroy et al., 1998) e mapeado no cromossomo 4p14 (Edwards et al., 1991).

O quadro clínico é similar à DP e a idade de início dos sintomas dos dois irmãos afetados era 49 e 51 anos. Não há estudos anátomo-patológicos nessa família (Leroy et al., 1998).

A enzima UCHL-1 participa do sistema ubiquitina-proteassoma atuando na desubiquitinação da proteína ubiquitina polimérica em monomérica (Ross e Pickart, 2004), e esta última forma, uma vez reciclada, pode ser utilizada em novos processos de ubiquitinação de substratos protéicos.

LRRK2 (PARK8)

A forma AD de parkinsonismo familiar causada por alteração no gene *PARK8* foi primariamente descrita em uma família Japonesa por Funayama et al. (2002). Essa família, também conhecida como família de Sagamihara, foi acompanhada pelos autores durante 20 anos e apesar da idade média de início ser um pouco mais cedo (51 anos) as características clínicas em nada diferiam daquelas da DP. Porém, o estudo anátomo-patológico de 4 membros da família revelou degeneração nigral pura, sem a presença de CL.

Em 2004, dois grupos independentes mapearam a mutação no gene *LRRK2* (*Leucine-rich repeat kinase 2*) (Paisan-Ruiz et al., 2004 e Zimprich et al., 2004). O gene *LRRK2* tem 51 exons e codifica uma proteína grande, de 2527 aminoácidos, que foi denominada Irrk2 ou dardarina, termo derivado de *dardara* que em basco significa tremor (Paisan-Ruiz et al., 2004). A Irrk2 e a Irrk1 são membros de uma recém descoberta família de proteínas quinase e apresentam uma seqüência similar à das tirosina e das serina-treonina quinases (Shen, 2004).

A seqüência da proteína Irrk2 compreende múltiplos domínios: 12 repetições ricas em leucina (LRR), um domínio Ras/ pequenas GTPase superfamília, um domínio tipo tirosina quinase e um domínio WD40 (Mata et al., 2006). Bosgraaf e van Haastert (2003) denominaram o domínio Ras/GTPase de Roc (*Ras of complex protein*). Dessa forma, a proteína Irrk2 faz parte do grupo das famílias ROCO, que são parte da família Ras/GTPase que são compostas por dois domínios importantes: Roc

e COR (C-terminal de Roc) além dos outros domínios acima descritos (Figura 2.1).

Figura 2.1: Representação esquemática da proteína Lrrk2

Representação esquemática da proteína Lrrk2. As mutações por substituições simples de aminoácidos descritas até o momento estão ilustradas. ANK= região de repetição de anquirina, COR= terminal C do Roc, Ex= exon, LRR= repetições rica em leucina, Roc= complexo de Ras (GTPase). Extraído de Mata et al. (2006).

A investigação de um número grande de famílias com parkinsonismo com padrão de herança AD por Di Fonzo et al. (2006) mostrou que as mutações mais comuns do gene *LRRK2* são, em ordem decrescente de frequência, Gli2019Ser, Arg1441Cis e Ile1371Val.

Esses dados estão de acordo com os achados de outros grupos e a frequência da mutação nesse gene pode variar de 3 a 41% (Brice, 2005; Lesage et al., 2006; Ozelius et al., 2006), sendo que na maioria dos estudos a frequência oscila entre 5 e 6% (Di Fonzo et al., 2005; Cookson et al. 2005). Nos casos esporádicos de DP, acredita-se que a mutação Gli2019Ser deve ser responsável pela doença em 1% dos casos (Cookson et al., 2005).

As mutações no gene *LRRK2* são as maiores responsáveis por parkinsonismo familiar com herança AD. Berg et al. (2005), observaram uma frequência de 13% em estudo na população alemã. No entanto, a mutação

Gli2019Ser, não foi encontrada neste grupo. A análise de haplótipo evidencia que a mutação Gli2019Ser tem um ancestral comum (Goldwurm et al., 2005). Há indícios de que a penetrância da mutação *LRRK2* é idade dependente (Di Fonzo et al., 2005)

O quadro clínico dos pacientes portadores de mutações no gene *LRRK2* é muito similar ao da DP. Na série descrita por Di Fonzo et al. (2006) a idade de início dos sintomas dos portadores da mutação Gli2019Ser variava 38 a 68 anos (média de 54,2 anos), dos portadores de Arg1441Cis entre 63 a 65 anos e dos portadores de Ile1371Val entre 41 a 72 anos. A autópsia realizada em um caso da série descrita por Berg et al. (2005) revelou a presença de CL. Esse achado também foi descrito em um caso descrito por Zamprich et al. (2004).

2.2 Parkinsonismo de transmissão autossômica recessiva

PARK2

O gene *PARK2* (6q25-q27) tem padrão de transmissão AR e foi primariamente descrito em famílias japonesas (Kitada et al., 1998). Estudos subsequentes comprovaram a presença desta mutação em grupos étnicos diversos (Rawal et al., 2003).

O gene tem mais que 1 Mb de extensão, 12 exons, codifica a proteína parkina de 465 aminoácidos e se expressa no cérebro e em outros tecidos (Dawson e Dawson, 2003; Bonifati et al., 2004a). A parkina é uma E3

ubiquitina ligase e, portanto tem um papel ativo no sistema ubiquitina-proteassoma que é responsável pela remoção e a reciclagem de proteínas celulares (Dawson e Dawson, 2003).

Uma vez que a doença tem padrão AR, a perda da função da proteína parkina leva ao aumento dos substratos que são reconhecidos pela sua função ubiquitina ligase (Ciechanover, 2001). Até o presente momento, os substratos identificados que se ligam à parkina são: CDCrel-1 (*cell division control-related protein 1*), Pael-R (*parkin-associated endothelin receptor-like receptor*), Sp22 (*O-glycosylated form of α -synuclein*), sinfilina-1, sinaptotagmina XI, SEPT5_v/CDCrel-2, ciclina E, subunidade p38 do complexo aminoacil-tRNA sintetase e / tubulina. Porém somente três substratos foram encontrados até o momento acumulados em cérebro de pacientes com parkinsonismo pela mutação do gene *PARK2* e são: CDCrel-1, Pael-R e Sp22. (Kubo et al., 2006).

Uma outra função recentemente descrita da parkina é a de catalisar a ubiquitinação ligada à lisina 63 (K63), que não é reconhecida pelo proteassoma, mas ao contrário, nesse processo a ubiquitinação envolve processos celulares diversos como a endocitose. A contribuição dessa disfunção na gênese do parkinsonismo relacionado ao gene *PARK2* ainda é desconhecida (Kubo et al., 2006).

A proteína parkina também participa na regulação da função mitocondrial por mecanismos ainda não elucidados (Abou-Sleiman et al., 2006). Modelos genéticos de *Drosophila PARK2-null* apresentavam mitocondriopatia com redução da longevidade, dificuldade à locomoção

(degeneração muscular) e esterilidade masculina por defeito da espermatogênese (Greene et al., 2003).

Posteriormente Whitworth et al. (2005) mostraram que essas *Drosophilas* apresentavam neurodegeneração dopaminérgica e que essa degeneração estava relacionada com perda de função do gene *Gutational S Transferase S1*. Por outro lado, o aumento da expressão desse gene nas células dopaminérgicas minimiza a perda neuronal.

O aumento da expressão da proteína parkina protege células cultivadas da apoptose induzida por mitocondriopatia além de exercer um efeito citoprotetor contra diversos agentes tóxicos (Kubo et al., 2006).

Na revisão de Hedrich et al. (2004) pelo menos 95 mutações diferentes foram identificadas até a ocasião da publicação e incluíam 40 rearranjos de exons (26 deleções e 14 multiplicações), 43 substituições simples de base e 12 pequenas deleções ou inserções de bases.

As mutações mais comuns em ordem decrescente de frequência eram: deleção do exon 4, do exon 3 e do exon 3 para 4. Os pontos principais para pequenas mutações encontram-se nos exons 2 (255/256delA é a mais freqüentes) e 7. No exon 7 os dados também sugerem que há o fenômeno do ancestral comum na mutação pontual mais comum desse exon, a 924C>T.

Mutações do gene *PARK2* são encontradas em cerca de 50% das famílias com padrão de transmissão autossômico recessivo e início dos sintomas abaixo de 45 anos. Em casos esporádicos de início precoce (< 45

anos) a frequência cai para 15 a 20% (Lucking et al., 2000; Periquet et al., 2003).

O quadro clínico foi inicialmente caracterizado como parkinsonismo de instalação precoce (<40 anos), com presença de complicações motoras secundárias ao uso de levodopa e curso lentamente progressivo e, portanto, muitas vezes indistinguível da DP. No entanto, estudos posteriores mostraram que o fenótipo da doença é mais amplo (Kubo et al., 2006). A Tabela 2.1 resume os achados clínicos dos pacientes com mutação do gene *PARK2*.

Tabela 2.1: Quadro clínico de pacientes com mutação do gene *PARK2*

Idade de início < 40 anos de idade
Cognição preservada
Distonia do pé freqüente
Instabilidade postural, retropulsão (em alguns casos), <i>freezing</i> e festinação precoce
Excelente resposta à levodopa com complicações motoras e psiquiátricas tardias devido ao uso do fármaco
Excelente resposta a anticolinérgicos em alguns casos
Curso lento e benigno
Fenótipos atípicos incluem:
Início tardio mimetizando DP
Psicose, ataques de pânico, depressão, hipersexualidade, comportamento obsessivo-compulsivo
Distonia induzida por exercício
Predomínio de síndrome rígido-acinética
Distonia focal (“câimbra do escrivão”, cervical)
Neuropatia autonômica ou periférica
Disfunções do trato cerebelar ou piramidal

Adaptado de Kubo et al., 2006

A idade de início é o principal fator preditivo para a presença de mutação do gene *PARK2*. Quanto mais precoce a instalação, maior a

chance de apresentar mutação desse gene. Algumas peculiaridades dos portadores de mutação no gene *PARK2* são: distonia no início do quadro (principalmente do pé), hiperreflexia, depressão, ataxia, alterações comportamentais e neuropatia periférica (tabela 2.1). Essa variação fenotípica se deve em parte às diferentes mutações no gene *PARK2* e influências ambientais (Lohmann et al., 2003).

Poucos cérebros de pacientes com *PARK2* foram estudados até o presente momento. Os achados típicos incluem perda neuronal e gliose na substância negra *pars compacta* e loco cerúleo. Há relatos de alterações que se estendem para substância negra *pars reticulada*, vias espinocerebelares e inclusões com proteína tau. É interessante notar que não se encontra CL nesses pacientes (Yamamura et al., 2000; van de Warrenburg et al., 2001). Entretanto, CL foram encontrados em um caso descrito por Farrer et al. (2001) em que o paciente era heterozigoto composto. Uma das mutações era a deleção do exon 3 e a outra era uma mutação com troca de aminoácido Arg275Trp.

Estudos recentes indicam que poucas mutações do gene *PARK2*, incluindo a Arg275Trp, induzem a formação de agregados intracitoplasmáticos em tecidos cultivados. Acredita-se que as mutações *PARK2* levem a proteínas que são rapidamente degradadas, o que gera a perda funcional. A mutação Arg275Trp preserva a sua atividade ubiquitina ligase e desta forma gera degeneração celular pela toxicidade, uma vez que é capaz de formar inclusões intracitoplasmáticas, e não pela perda de função (Chung et al., 2001; Cookson et al., 2003).

Em um número considerável de estudos há descrição de portadores heterozigotos da mutação do gene *PARK2* com parkinsonismo, porém nestes casos a idade de instalação dos sintomas tende a ser mais tardia e o quadro clínico similar ao da DP (Foroud et al., 2003). Pelo fato da doença ter padrão de herança AR, a expressão do parkinsonismo nestes pacientes pode ser pelo mecanismo de haploinsuficiência, efeito dominante negativo ou interação com mutações desconhecidas localizadas em outros *loci*. Outra possibilidade é que o portador heterozigoto é mais susceptível a fatores ambientais para o desenvolvimento de parkinsonismo (Kubo et al., 2006).

PARK6

Valente et al. (2001) descreveram uma família siciliana com padrão de herança autossômica recessiva em que o defeito genético estava no *locus* 1p35-p36. Posteriormente, Valente et al. (2004a) identificaram a mutação que segregava a doença no gene *PARK6*. Mutações desse gene também foram encontradas em famílias européias e asiáticas (Bonifati et al., 2005).

O gene *PARK6* tem oito exons, 18 Kb, e codifica uma proteína quinase, PINK1 (*PTEN-induced kinase 1*), com algum grau de homologia com a serina-treonina quinase da família Ca/calmodulina. A proteína codificada tem localização intramitocondrial e confere efeito protetor contra o estresse oxidativo. (Healy et al., 2004).

A maioria das mutações encontradas está dentro do domínio funcional da proteína PINK1 e geralmente determinando substituições simples de aminoácidos (Valente et al., 2004b; Hatano et al., 2004). Porém, Rohe et al.

(2004), descreveram uma mutação por inserção, a 1573_1574 - insTTAG, que está localizada fora do domínio funcional da proteína (domínio serina-treonina da proteína quinase). Curiosamente, o quadro clínico deste indivíduo, recessivo para a mutação, assemelha-se ao de pacientes com mutações de *PARK2* o que sugere que o espectro fenotípico de *PARK6* está relacionado aos achados genotípicos.

A frequência da mutação do gene *PARK6* é estimada em 3,3% na população italiana com PP de início precoce enquanto que na população asiática a frequência aumenta para 15% (Kubo et al., 2006).

Em casos de PP de início precoce, mas de ocorrência esporádica, heterozigotos para mutações no gene *PARK6* foram encontrados em 5% nas séries estudadas (Valente et al, 2004b; Bonifati et al., 2005). Da mesma forma que nos casos de *PARK2* a expressão da doença nos heterozigotos ainda precisa ser elucidada.

O quadro clínico é muito similar ao da DP, mas o início das manifestações é precoce (entre 37 a 47 anos). Há raras descrições de alterações comportamentais ou psiquiátricas como depressão, ansiedade, alucinação e demência (Kubo et al., 2006). Não se tem conhecimento das alterações anátomo-patológicas (Valente et al., 2004b).

Bonifati et al. (2005) investigaram uma grande população de pacientes com PP de início precoce e notaram que havia uma grande variabilidade fenotípica nos pacientes com mutação no gene *PARK6*, que incluía distonia no início do quadro, instalação simétrica dos sintomas e ansiedade. Um caso apresentava neuropatia periférica.

PARK7

Mutações no gene *PARK7* foram identificadas em duas famílias (italiana e holandesa) com padrão de herança AD em 2003 por Bonifati et al. O gene *PARK7* tem 24 Kb e oito exons. A sua expressão ocorre em todos os tecidos cerebrais. A proteína codificada pelo gene, DJ-1, tem 189 aminoácidos, pertence à família ThiJ/ Pfp/ DJ-1 e sua função é ainda desconhecida. Acredita-se que ela seja importante em situações de estresse celular e a patogênese decorre pela perda da função da proteína mutante (Bonifati et al., 2004b).

A proteína DJ-1 está envolvida em múltiplas funções, porém a mais importante é a de antioxidante. Quando exposto ao peróxido de hidrogênio (H_2O_2), ocorre uma modificação no resíduo cisteína da proteína DJ-1 que passa a atuar como um sinalizador de estresse oxidativo, ativando reações antiapoptóticas. Estudos indicam que a perda da função da proteína leva ao aumento de espécies reativas de oxigênio (ROS – *reactive oxygen species*) e suscetibilidade para degeneração de células dopaminérgicas (Abou-Sleiman et al., 2006).

Indícios sugerem que em situação de estresse celular, a proteína DJ-1 interage com a proteína parkina, levando à suposição de que ambas as proteínas devam atuar em mecanismos similares de neuroproteção (Moore et al., 2005).

A frequência de mutações no gene *PARK7* entre os casos de parkinsonismo de início precoce é baixa e está em torno de 1% a 2% (Clark et al., 2004; Abou-Sleiman et al., 2006).

O quadro clínico é similar ao da DP, mas a idade de início é em torno dos 30 anos de idade. Além disso, há relatos da presença de distonia, alterações psiquiátricas e comportamentais. Não se sabe da ocorrência ou não de CL porque não há estudos anátomo-patológicos até o momento (Kubo et al., 2006).

ATP13A2 (PARK9)

Em 1994, Najim Al-Din et al. descreveram cinco irmãos, filhos de pais consangüíneos, que apresentavam quadro clínico de parkinsonismo atípico de início precoce (entre 12 a 16 anos) com padrão de herança AR. As atipias incluíam paralisia supranuclear do olhar vertical, espasticidade e demência. O curso era rapidamente progressivo, mas os pacientes apresentavam resposta à levodopa e moderada regressão dos sintomas. A ressonância magnética evidenciava atrofia dos globos pálidos e nos quadros mais avançados, atrofia generalizada. Os autores nomearam esta nova entidade nosológica de síndrome de Kufor-Rakeb, pois este é o nome da comunidade no norte da Jordânia onde residia a família.

Posteriormente, Hampshire et al. (2001) mapearam o defeito genético no braço curto do cromossomo 1 (1p36) numa região de 9 cM entre os marcadores D1S436 e D1S2853.

Em 2005, quatro afetados da família jordaniana foram reexaminados por Williams et al., que reforçaram os aspectos atípicos dessa doença e descreveram a presença de um outro distúrbio de movimento caracterizado por movimentos periorais e nos dedos das mãos similares a mioclonias que

denominaram de mini mioclonias de face-fauce-dedos (*facial-faucial-fingers mini-myoclonus*), além de alucinação e distonia oculógira.

Originalmente, Najim Al-Din et al. (1994) acreditavam que a família de Kufor-Rakeb assemelhava-se à síndrome pálido-piramidal descrita por Davidson em 1954 (Davidson, 1954 apud Williams et al., 2005), em que os pacientes apresentavam parkinsonismo juvenil ou de início precoce e quadro piramidal bilateral. Na revisão posterior de Williams et al. (2005) os autores analisaram a família jordaniana e observaram que ela apresentava algumas peculiaridades, mas não era similar aos descritos por Davidson em que os pacientes apresentavam um quadro clínico heterogêneo, pois além dos sinais piramidais e extrapiramidais, alguns tinham comprometimento cerebelar, flutuação diurna ou ausência de tremor. Segundo os mesmos autores, esses achados podem significar que diferentes doenças foram englobadas na síndrome pálido-piramidal ou é esta uma entidade nosológica com grande variedade fenotípica.

Embora a síndrome de Kufor Rakeb esteja classificada dentre os parkinsonismos hereditários pela HUGO (*The Human Genome Organisation*), ela apresenta características clínicas peculiares que não estão presentes na DP. Williams et al. (2005), sugerem classificá-la como parkinsonismo hereditário raro com resposta satisfatória ao uso de levodopa, de início subagudo com progressão para restrição motora grave e limitação para as atividades de vida diária e envolvimento de áreas dos núcleos da base, vias de controle do olhar vertical e córtex cerebral.

Ramirez et al., em 2006, identificaram uma família chilena com as mesmas características clínicas e genéticas da família de Kufor-Rakeb e conseguiram mapear uma mutação no gene *ATP13A2*. O gene codifica uma ATPase transmembrana do tipo P. A forma selvagem é ubiquamente expressa mas principalmente no cérebro. Além disso, a proteína selvagem *ATP13A2* foi encontrada em lisossomos, a forma mutante por sua vez, no retículo endoplasmático. Esse achado pode significar um prejuízo na degradação protéica pelo sistema lisossomal.

2.3 Etiopatogenia da Doença de Parkinson: Contribuição da genética

As formas monogênicas de PP familiar contribuíram muito para o esclarecimento dos mecanismos de morte celular. As mutações nos genes *SNCA* e *PARK2* mostraram a importância da proteína α -sinucleína e do sistema ubiquitina-proteassoma, que serão revistos a seguir.

Proteína α -sinucleína

A proteína α -sinucleína desempenha um importante papel na DP por várias razões: 1) a α -sinucleína está presente nos CL (Spillantini et al., 1997); 2) mutações no gene da α -sinucleína estão associadas a formas familiares raras de parkinsonismo (Polymeropoulos et al., 1997); 3) a

expressão de α -sinucleína em modelos de camundongos transgênicos (Giasson et al., 2003; Hashimoto et al., 2004) e *Drosophila* mimetizam vários aspectos da DP (Feany e Bender, 2000).

A proteína α -sinucleína tem 140 aminoácidos e foi originalmente identificada em cérebro humano como a proteína precursora do componente não β -amilóide das placas amilóides da doença de Alzheimer (Hashimoto et al., 2004). Ela foi denominada sinucleína porque os achados iniciais indicavam que a proteína estava presente nas sinapses (Snyder e Wolozin, 2004).

A α -sinucleína é membro de uma grande família protéica da qual fazem parte as proteínas homólogas β -sinucleína, γ -sinucleína e sinoretina. Embora elas sejam ubíquas, a α -sinucleína é particularmente abundante nas sinapses cerebrais e representa cerca de 1% das proteínas cerebrais (Snyder e Wolozin, 2004). Dentre as três formas a α -sinucleína é a única que contém um domínio altamente amiloidogênico e por isso forma fibrilas (Lee e Trojanowski, 2006).

Apesar da similaridade entre as três proteínas sinápticas, as suas funções ainda são desconhecidas. Evidências indicam que a α -sinucleína regula o nível ou o metabolismo da β -sinucleína uma vez que em camundongos transgênicos, a α -sinucleína inibe a agregação da β -sinucleína (Lee e Trojanowski, 2006).

A proteína é encontrada no citoplasma de modo não dobrado e tem um domínio de ligação com ácidos graxos. A ligação com os lipídios deve ter um papel importante para o funcionamento protéico (Snyder e Wolozin, 2004).

Lotharius e Brundin (2002), sugerem que anormalidades na regulação sobre os fosfolípidos e ácidos graxos promovem alterações nas vesículas que estocam a dopamina no neurônio pré-sináptico, resultando em liberação aberrante desse neurotransmissor no citoplasma conseqüentemente causando estresse oxidativo ou disfunção metabólica neuronal.

Estudo recente sugere que a proteína também está envolvida no trânsito de substratos dentro dos retículos endoplasmáticos, complexo de Golgi e em fungos, a interrupção deste tráfego gera um aumento de expressão da α -sinucleína (Lee e Trojanowski, 2006).

A molécula da α -sinucleína é altamente estável e liga-se a várias proteínas promovendo mudanças conformacionais que podem gerar agregados patológicos (Hashimoto et al., 2004).

Acredita-se que o acúmulo de α -sinucleína pode levar à neurodegeneração. Esse fato é embasado em estudos genéticos em que as mutações do gene da α -sinucleína produzem doenças neurodegenerativas. Tanto a mutação Ala30Pro quanto a Ala53Tre aceleram a agregação da proteína anômala (Conway et al., 2000).

Os camundongos transgênicos que expressam a mutação Ala53Tre além de desenvolver alterações motoras graves, apresentam inclusões intracitoplasmáticas contendo α -sinucleína, similares aos achados anátomo-patológicos em humanos (Giasson et al., 2003). Pode-se concluir que a mutação no gene SNCA leva à formação de filamentos tóxicos da proteína anômala formando inclusões neuronais que provocam degeneração neuronal.

Além da mutação genética, outros fatores promovem a agregação da α -sinucleína e incluem: disfunção mitocondrial, proteína amilóide, estresse oxidativo, oxidação direta da α -sinucleína e neurotoxinas como a MPTP (Hashimoto et al., 2004).

A proteína α -sinucleína se liga ao proteassoma e provavelmente exerce uma função modulatória sobre esse complexo protéico. Agregados de α -sinucleína inibem a atividade do proteassoma 26S dez mil vezes mais do que a forma monomérica (Snyder e Wolozin, 2004).

A degradação da α -sinucleína é realizada pelas vias ubiquitina-proteassoma 26S dependente e independente. A deficiência do sistema proteassomal leva a acúmulos da proteína que podem provocar a degeneração neuronal. Apesar disso, alguns estudos mostram que neurônios tratados com inibidores proteassomais com subsequente formação de inclusões com α -sinucleína têm maior taxa de sobrevivência que os neurônios que não desenvolvem as inclusões (Snyder e Wolozin 2000).

Outras formas de degradação da α -sinucleína são conhecidas. As proteínas de meia vida curta são geralmente decompostas pelo sistema proteassomal ao passo que, as proteínas de meia vida longa pela via autofágica dentro dos lisossomos. Uma parcela das proteínas citoplasmáticas é reconhecida pela chaperona hsc70 e degradada nos lisossomos num processo conhecido como autofagia chaperona mediada. (Cuervo et al., 2004).

Cuervo et al. (2004) demonstraram que a α -sinucleína selvagem é eficientemente degradada nos lisossomos pelo processo chaperona

mediado, mas as proteínas mutantes (Ala 53Tre e Ala30Pro) são pobremente degradadas por essa via. Esse fato gera a disfunção do sistema lisossomal o que aumenta a concentração dessas proteínas anômalas e sua posterior agregação. Além do bloqueio da sua decomposição elas também impedem a degradação de outras proteínas de meia vida longa pelos lisossomos contribuindo para o estresse celular.

Em resumo, os mecanismos da neurodegeneração devido a alterações da α -sinucleína ainda não estão elucidados, mas várias hipóteses foram levantadas. A proteína localiza-se no terminal pré-sináptico e liga-se à membrana sináptica. O seqüestro de α -sinucleína em agregados ou fibrilas de amilóides na DP impede-a de exercer a sua função e possivelmente afeta outras proteínas envolvidas no processamento das sinapses. Nos casos das mutações do gene *SNCA*, há alterações conformacionais da estrutura da α -sinucleína que leva a um aumento da sua fibrilização e conseqüente neurotoxicidade. Alguns experimentos, porém mostram que pequenos oligômeros pré-fibrilares da α -sinucleína são os verdadeiros fatores neurotóxicos que levam à degeneração neuronal por alterar a permeabilidade de mitocôndrias e outras organelas. Além disso, α -sinucleínas anômalas nos retículos endoplasmáticos e complexo de Golgi levam ao bloqueio de tráfego protéico e morte celular (Lee e Trojanowski, 2006). A figura 2.2 mostra o modelo de agregação da α -sinucleína.

Figura 2.2: Agregação da α -sinucleína

Modelo esquemático da agregação da proteína α -sinucleína. A proteína truncada converte-se em pequenos oligômeros patológicos que se agregam, formam fibrilas e se depositam nos corpúsculos de Lewy. As alterações genéticas (mutações do gene SNCA) ou ambientais (ex. pesticidas) aceleram esse processo de modo que os sistemas de controle de qualidade celular (chaperonas, sistema ubiquitina-proteossoma, fagossomos/ lisossomos) não conseguem prevenir, reverter ou eliminar as proteínas desdobradas e conseqüentemente formam agregados ou fibrilas de amilóides. O acúmulo dessas proteínas anômalas leva à degeneração neuronal por mecanismos diversos (estresse oxidativo, interrupção do transporte axonal, sequestro de proteínas, disfunção mitocondrial, disfunção sináptica, inibição do sistema ubiquitina-proteossoma). Extraído de Lee e Trojanowski, 2006.

Sistema ubiquitina-proteassoma

A remoção e a reciclagem de proteínas no citoplasma são muito importantes para a manutenção da saúde celular. Um dos mecanismos mais importantes para a modificação do substrato protéico e sua posterior degradação pelos proteassomas é a ubiquitinação. A ubiquitina é um polipeptídeo de 76 resíduos de aminoácidos. Neste processo, as proteínas alvo são modificadas pelas ubiquitinas ou proteínas tipo-ubiquitina. A remodelação da superfície dessas proteínas afeta, entre outras propriedades, sua estabilidade, interação com outras proteínas, atividade e localização subcelular (Ciechanover, 2006)

A conjugação protéica com a ubiquitina ou proteínas tipo-ubiquitina também é a base para diversas funções não proteolíticas como modulação da dinâmica da membrana celular, ativação de mecanismos regulatórios de transcrição, ou direcionamento da proteína alvo para reações intracelulares subjacentes. Desta forma, a ubiquitinação é um processo controlado e altamente complexo de múltiplas etapas.

A degradação protéica pela via ubiquitina-proteassoma envolve duas etapas: sinalização da proteína alvo e ligações covalentes com múltiplas moléculas de ubiquitina resultando em uma cadeia poliubiquitinada; degradação da proteína alvo pelo complexo protéico proteassoma 26S e liberação das moléculas de ubiquitina para reutilização.

A conjugação de ubiquitina com o substrato protéico ocorre em três etapas. Inicialmente, a enzima ativadora de ubiquitina, também denominada de E1, ativa a molécula de ubiquitina por meio de uma reação ATP

dependente para gerar um substrato intermediário de alta energia. A seguir, uma das enzimas conjugadoras de ubiquitina, E2, transfere a ubiquitina ativada por E1 para um substrato específico, que é uma enzima proteína ubiquitina ligase, ou E3 (Figura 2.3).

Figura 2.3: Sistema de ubiquitinização

Extraído de: <http://en.wikipedia.org/wiki/Image:Ubiquitylation.png>

E1: enzima ativadora de ubiquitina; E2: enzima conjugadora de ubiquitina; E3: enzima proteína ubiquitina ligase; Ub: ubiquitina; Substrate: substrato protéico.

Existem várias classes de enzimas E3, a maioria tem o anel RING (*Really Interesting New Gene*), localizado no C-terminal e cuja função é recrutar a enzima E2 (Snyder e Wolozin, 2004). A E3 catalisa a última etapa do processo de conjugação: une covalentemente a ubiquitina ao substrato protéico. Aproximadamente 100 subtipos de E3 já foram identificados no genoma humano. A proteína parkina é uma enzima E3 e o seu terminal

amino (N) se liga à subunidade RPN10 do proteassoma 26S (Abou-Sleiman et al., 2006).

O proteassoma é uma protease multicatalisadora que degrada proteínas poliubiquitinadas e as transforma em pequenos peptídeos. Três diferentes vias proteassomais foram identificadas até o presente momento: 20S proteassoma ubiquitina-independente; 26S proteassoma ubiquitina-independente e 26S proteassoma ubiquitina-dependente (Baumeister et al., 1998). Embora a via 26S ubiquitina dependente seja bem caracterizada, não se conhece muito bem o mecanismo da via independente (Snyder e Wolozin, 2004).

Tanto a 26S ubiquitina dependente como a independente têm o centro 20S que realiza a função catalítica e duas coberturas 19S, que são partículas regulatórias. A estrutura 20S tem estrutura tubular composta de quatro anéis, dois alfa e dois beta, que por sua vez são compostas de sete subunidades distintas. A ação catalítica ocorre nas subunidades beta.

As partículas 19S têm função regulatória, pois reconhecem as proteínas ubiquitinadas. Além disso, abrem um orifício no anel , permitindo a entrada do substrato na câmara proteolítica. Uma vez que a proteína não conseguiria entrar no estreito canal proteassomal, acredita-se que essas organelas têm a função de desdobrar o substrato protéico permitindo a sua entrada na estrutura 20S (Figuras 2.4 e 2.5).

Figura 2.4: Estrutura do proteassoma 20S de *Thermoplasma acidophilum*

(a) Modelo derivado de mapeamento atômico do proteassoma de *Thermoplasma*. As subunidades α formam o anel externo heptamérico e as β o interno. Esta estrutura arquitetônica é conservada desde *Thermoplasma* a células eucarióticas.
 (b) Esquema do modelo tridimensional da 20S, as hélices coloridas indicam as subunidades de cada anel. A escala da barra é de 10 nm.

Extraído de Baumeister et al., 1998.

Uma vez degradada a proteína em pequenos peptídeos, esses são liberados no citoplasma. A enzima UCH-L1, (ubiquitina carboxi terminal esterase L1), participa no processo de desubiquitinação (Ross e Pickart, 2004) e uma vez reciclados, os monômeros de ubiquitina podem ser utilizados em novos processos de ubiquitinação de substratos protéicos.

Figure 2.5: Sistema ubiquitina-proteassoma

Sistema ubiquitina-proteassoma. UCH: ubiquitina C-terminal esterase; Ub: ubiquitina; 26S proteassoma: 26S proteassoma ubiquitina dependente com duas coberturas 19S e o centro 20S; substrato protéico está representado em vermelho. E1, E2 e E3: enzimas ubiquitina ativadora, conjugadora e ligase respectivamente. Extraído de Ross e Pickart, 2004.

2.4 Mecanismo do parkinsonismo

Os mecanismo pelos quais as mutações genéticas levam ao parkinsonismo ainda são desconhecidos, contudo frente ao conhecimento que se tem até o presente momento pode-se construir um modelo de neurodegeneração que será descrito a seguir (Farrer, 2006).

As mutações no gene SNCA sejam as substituições simples de aminoácido ou multiplicação genômica, levam a formação de proteína -sinucleína (monômero) anômala ou ao aumento da sua expressão que se acumula no citoplasma. Este acúmulo promove a oligomerização da -sinucleína que é tóxica para a célula. O neurônio degrada a proteína via sistema ubiquitina-proteassoma, sistema endossoma-lisossomal ou formando um agregado fibrilar de alto peso molecular. A proteína -sinucleína participa na formação de vesículas e na neurotransmissão de dopamina, mas por ser anômala, impede a sinapse e leva ao acúmulo desse neurotransmissor que é tóxico e conseqüente formação de espécies reativas de oxigênio que por sua vez provocam a morte celular.

As proteínas parkina e DJ-1 participam do sistema ubiquitina-proteassoma e o defeito nestas proteínas pode diminuir a eliminação dos agregados tóxicos de -sinucleína. A proteína DJ-1 também tem função antioxidante e sua anomalia pode facilitar a fibrilização da -sinucleína mal-formadas. Na DP os agregados de -sinucleína acumulados nos citoplasma e axônios compõem os CL.

A proteína UCLH1 (ubiquitina carboxi terminal esterase L1), que tem atividades hidrolase e ligase, atua no sistema ubiquitina proteassoma, na via endossoma-lisossomal e na formação do CL. A sua função é prover monoubiquitina para a proteína E3 ligase (parkina) e evitar que a ubiquitina seja degradada pela via endossoma-lisossomal.

As mutações dos genes *PARK6*, *PARK7* e *PARK2* resultam na disfunção da mitocôndria, produtora de ATP, que é essencial para o funcionamento do sistema ubiquitina proteassoma.

Para a eliminação dos agregados de α -sinucleína é também necessária a preservação da citoarquitetura, o que inclui os microtúbulos. A proteína tau estabiliza a função dos microtúbulos. A proteína Lrrk2 (quinase com repetição rica de leucina) é importante na manutenção do tráfego intracelular e citoarquitetura a sua disfunção pode conseqüentemente levar ao acúmulo de proteína tau e formar agregados que também são citotóxicos.

Figura 2.6: Modelo de parkinsonismo

Adaptado de Farrer, 2006.

MÉTODOS

MÉTODOS

3.1 Pacientes

Seleção dos Pacientes

A seleção dos casos foi feita entre pacientes acompanhados no Ambulatório do Grupo de Estudo de Distúrbios do Movimento da Clínica Neurológica do Hospital das Clínicas da FMUSP e seus familiares, segundo critérios específicos de inclusão e exclusão.

O estudo foi aprovado pela Comissão de ética para Análise de Projetos de Pesquisa (CAPPesq) da Diretoria Clínica do Hospital das Clínicas do Hospital das Clínicas da FMUSP e todos os pacientes e familiares incluídos no trabalho assinaram o Termo de Consentimento Livre e Esclarecido.

Critérios diagnósticos e escala de avaliação

O diagnóstico de PP seguiu os critérios estabelecidos pela *The United Kingdom Parkinson's Disease Society Brain Research Centre, Institute of Neurology, London* (Hughes et al., 1992). Os critérios maiores são a presença de bradicinesia e uma das manifestações a seguir: tremor de repouso, rigidez e instabilidade postural. Os critérios auxiliares de suporte incluem três dos seguintes itens: início unilateral, tremor de repouso, progressão evidente, persistência da assimetria do quadro, excelente

resposta à levodopa, discinesias acentuadas levodopa induzida, resposta à levodopa por mais de 5 anos, curso clínico de mais de 10 anos.

No exame neurológico, pacientes com hiperreflexia dos reflexos miotáticos foram admitidos, mas pacientes com outras manifestações neurológicas que não o parkinsonismo foram excluídos do estudo. Foram utilizadas as seguintes escalas de avaliação *Unified Parkinson's Disease Rating Scale* (UPDRS), bloco motor - parte III e a escala de Hoehn e Yahr (Fahn e Elton, 1987).

Critérios de Inclusão:

1. Parkinsonismo primário
2. Ausência de outros sinais neurológicos
3. Manifestações têm boa resposta a levodopa ou agonistas dopaminérgicos
4. Instalação antes ou aos 40 anos de idade.
5. História familiar positiva para parkinsonismo primário

Critérios Auxiliares:

1. Consangüinidade
2. Flutuações motoras (discinesia induzida por levodopa)
3. Distonia no início, ou antes, da introdução de drogas dopaminérgicas
4. Curso lentamente progressivo

Crterios de Excluso:

1. Alteraes neurolgicas outras que no parkinsonismo
2. Uso de neurolptico previamente manifestao de PP
3. Alterao em exames de neuroimagem

Os pacientes selecionados foram submetidos aos seguintes procedimentos:

- A. Avaliao e obteno de dados clnicos.
- B. Assinatura do Termo de Consentimento Livre e Esclarecido.
- C. Coleta de 20 mL de sangue venoso em tubo com EDTA.

O sangue coletado foi submetido extrao de DNA de leuccito. Uma alquota do DNA extrado foi enviada para o Laboratrio de Gentica da Erasmus University, em Rotterdam, aos cuidados do Dr. Vincenzo Bonifati, que foi responsvel pela anlise gentica.

Nomenclatura das famlias

As famlias foram catalogadas conforme orientao do Laboratrio de Gentica da Erasmus University, com a sigla PDBR (Parkinson's Disease – Brazil), e os primeiros dgitos aps a sigla referem-se ao nmero da famlia e o dgito aps o ponto identifica o indivduo. Exemplificando: PDBR01.1 o indivduo 1 da famlia 1, PDBR01.2 o indivduo dois da famlia 1 e sucessivamente.

3.2 Extração de DNA de leucócitos

A extração de DNA genômico de leucócitos foi realizada a partir de sangue periférico. Foram coletados 20 mL de sangue venoso divididos em 2 tubos de 10 mL cada, contendo 25 mM EDTA (ácido etilenodiaminotetracético). O *pellet* leucocitário foi obtido por hemólise utilizando-se solução de lise (1mM NH_4HCO_3 , 114 mM NH_4Cl), com incubação a 4°C por 30 minutos, seguida de centrifugação do material a 4°C por 15 minutos a 3000 rotações por minuto (rpm) desprezando-se o sobrenadante. A centrifugação foi repetida nas mesmas condições e então o *pellet* de leucócitos foi suspenso em 6 mL de solução de lise de glóbulos brancos (10 mM Tris, 10 mM EDTA, 150 mM de NaCl), 120 μL de SDS 10% (Sigma), 80 μL de proteinase K (10 mg/mL) (Gibco BRL) e incubado durante 18 horas à 37°C. No dia seguinte, foi adicionado 2,4 mL de NaCl saturado. Essa solução foi agitada vigorosamente por 15 segundos e centrifugada por 15 min a 3000 rpm. O sobrenadante contendo o DNA desproteínizado foi transferido para um tubo limpo onde se adicionou 2 volumes de etanol absoluto gelado, e homogeneizado cuidadosamente por inversão. O DNA precipitado retirado do tubo, foi lavado duas vezes com etanol gelado a 70%, em seguida com etanol absoluto e seco por centrifugação a vácuo (SpeedVac System, Savant ISS100) durante 5 min, e ressuspenso em solução de TE pH 8 (Tris-HCl 10 mM, EDTA 0,1mM). O produto da extração foi armazenado a 4°C.

3.3 Investigação do gene *PARK2*

Para a análise de haplótipos, foram identificados marcadores STR (*Short Tandem Repeats*) do gene *PARK2*, utilizando-se *primers* (oligonucleotídeos inicializadores) marcados com fluorescência. As seqüências de DNA foram obtidas em um seqüenciador automático ABI3100 e analisadas com o *software* GeneMapper versão 3.0 (Applied Biosystems). Os haplótipos foram identificados baseando-se no menor número de recombinações.

Os exons 2 a 12 do gene *PARK2* foram amplificados de acordo com o protocolo previamente estabelecidos (Bertoli-Avella et al., 2005). O exon1 foi amplificado num fragmento de 357 pbs (pares de base). Para solução final de 20 μ L utilizou-se 1 x tampão TaKaRa GCII, 400 μ M cada de dNTP, 1 μ M de *primers forward* e , 1 unidade de LA Taq DNA polimerase (TaKaRa) e 25 ng de DNA genômico. As condições de PCR (reação em cadeia da polimerase) foram: desnaturação inicial de 7 min e 30 seg a 96°C; 35 ciclos de 30 seg de desnaturação a 96°C, 30 seg de anelamento dos *primers* a 68°C e 1 min e 30 seg de extensão a 72°C; extensão final de 5 min a 72°C. Os seguintes *primers* foram utilizados para a amplificação do exon 1: 5'-ctgggggcaggaggcgtgag-3' (*forward*), and 5'-ggacggcacgggcacttttg-3' (*reverse*).

Investigação da mutação IVS1+1G/T

O RNA total foi isolado de sangue periférico e o DNAC de dois homozigotos e um heterozigoto da família PDBR01 e de controles foram preparados de acordo com protocolos padrões. Dois fragmentos de DNAC do gene *PARK2* que compreendem os exons 1 a 3 (173 pbs) e 4 a 6 (238pbs) foram amplificados por meio de um protocolo de *touchdown* PCR, utilizando-se os seguintes *primers*: exons 1 a 3 5'-aggagaccgctggtgggag-3' (*forward*), e 5'-ccacctccttgagctggaag-3' (*reverse*); exons 4 a 6 5'-gtcaaagagtgcagccggg-3' (*forward*), e 5'-ctattgttgcatcaggtgc-3' (*reverse*). Um fragmento de 385 pbs do DNAC do gene *HPRT* (hipoxantina fosforibosil transferase 1) foi amplificado como controle, por meio dos seguintes *primers*: 5'-cgtgggtccttttcaccagcaag-3' (*forward*) e 5'-aattatggacaggactgaacgtc-3' (*reverse*).

Os produtos de PCR foram purificados com 2 L ExoSAP-IT (USB) durante 30 min a 37°C, seguidos de 10 min de inativação a 80 °C. Seqüências senso e anti-senso, foram obtidas por meio do Big Dye Terminator chemistry versão 3.1 (Applied Biosystems). Os fragmentos de DNA foram alocados em um seqüenciador automático e analisados com *DNA Sequencing Analysis* (versão 3.7) e o software SeqScape (versão 2.1) (Applied Biosystems).

As conseqüências das mutações foram analisadas de acordo com a seqüência de RNAm do gene *PARK2* depositada no Genbank (número de acesso NM_004562).

3.4 Investigação do gene *LRRK2* – mutação G2019S

Após a extração do DNA genômico de leucócitos de sangue periférico, os 51 exons do gene *LRRK2* foram amplificados pela técnica de PCR e seqüenciamento direto das seqüências senso e anti-senso. As reações de PCR foram realizadas em volume final de 25 μ L contendo 1 x tampão Invitrogen, 15 mmol/L $MgCl_2$, 0,01% de detergente W1, 25 μ mol/L de cada dNTP, 0,4 μ mol/L *primers forward* e *reverse*, 2,5 unidades de Taq DNA polimerase (Invitrogen) e 50ng do DNA genômico. As condições da PCR foram: desnaturação inicial de 5 min a 94°C; 30 ciclos de 30 seg de 30 desnaturações a 94°C; 30 seg de anelamento dos primers a 68°C e 1 min e 90 seg de extensão a 72°C. Os primers foram utilizados para a amplificação do exon 41 (primers e a sua temperatura de anelamento estão descritos na tabela <http://image.thelancet.com/extras/04let12084webtable.pdf>).

O seqüenciamento direto das duas fitas foi realizado com *Big Dye Terminator Chemistry* (versão 3.7) (Applied Biosystems). Os fragmentos foram colocados em um seqüenciador automático ABI3100 e analisados por *DNA Sequencing Analysis* (versão 3.7) e analisados com os *softwares* SeqScape (verão 2.1) (Applied Biosystems). A análise das possíveis conseqüências das mutações na estrutura tridimensional da proteína foi realizada de acordo com a seqüência de DNAC depositada no GenBank (número de acesso AY792511).

3.5 Investigação do gene *ATP13A2*

Os vinte e nove exons e limites íntron-exon do gene *ATP13A2* foram amplificados por meio de PCR. Para o seqüenciamento de alguns exons, *primers* internos adicionais foram utilizados. Todas as seqüências dos *primers* e as condições de PCR estão descritas nas tabelas 3.1 e 3.2. As reações de PCR foram realizadas em volume final de 25 μ L contendo 1 x tampão de PCR, 1,5 mM $MgCl_2$, 0,01% de detergente W1, 25 μ M de cada dNTP, 1,2 μ M *primers forward* e *reverse*, 2,5 unidades de Taq Platinum (Invitrogen) e 50 ng de DNA genômico. Para amplificação do exon 1, 4 a 5, 9 a 11 e 26 a 27, foi utilizada TakaRa LA Taq polimerase e tampão de PCR GC II (Takara Biomedicals) foram utilizados. O seqüenciamento direto de ambas as fitas foi realizado com o *Big Dye Terminator chemistry* versão 3.1 (Applied Biosystems). Os fragmentos foram condicionados em um seqüenciador automático ABI3100 e analisados com os softwares *DNA Sequencing Analysis* (versão 3.7) e *software Seq Scape* (versão 2.1). As mutações foram numeradas a partir da adenina do códon de tradução inicial ATG e as possíveis consequências das mutações na proteína foram avaliadas de acordo com a seqüência do gene *ATP13A2* (GenBank número de acesso NM_022089.1) e a seqüência protéica (número de acesso NP_071372.1).

Tabela 3.1: *Primers* e as condições de PCR para a amplificação dos fragmentos genômicos do gene *ATP13A2*

Exon	Forward 5' → 3'	Reverse 5' → 3'	Annealing T (total 35 cycles)	Fragment size (bp)
1	GCGTCAGCCCTGCTCCAGC	AGGATCCCCAACCAGGTCC	68°	299
2-3	GCCCAGGCTGATGTTTATTG	TCCCATGGAAAGTCAGTGG	70° → 60 (-1°/cycle), then 60°	590
4-5	CTTCCCACATCTGAGCATTGG	GAACATGAAGTCTGACTCTCC	70° → 60 (-1°/cycle), then 60°	626
6	GAGCACGTTTACACTTTTCAGG	GCCATGCCACCGTCTGTGC	70° → 60 (-1°/cycle), then 60°	306
7-8	CCTAGAGCCCATAGTCTCC	GGACAGCTGGTCTGGTTGC	70° → 60 (-1°/cycle), then 60°	542
9-10-11	TGGGCTGCCCTGGGGGACG	CAGGGCAAAGGGTTGGATGG	70° → 62 (-1°/cycle), then 62°	638
12	ACACAGAACTCTGGTTTGC	CACTCCACCTCTCCCAAGG	70° → 60 (-1°/cycle), then 60°	291
13-14-15	CTGTTAGGACTCCACACTGC	AGACATTTTCATCTGCCAGACC	70° → 66 (-1°/cycle), then 66°	859
16	CTCCAGCTCGAGTTCCTGG	TGGGCATAATAAGAGACCACC	70° → 60 (-1°/cycle), then 60°	433
17-18	AGAGTTTGAATTTGAGTCC	ACC TGGCAGGGACAGAGGCTGG	70° → 66 (-1°/cycle), then 66°	567
19-20	CCGAGGCCTACGTCAAAGG	TGGTGCCCCCTCTTCTCTGC	70° → 60 (-1°/cycle), then 60°	641
21-22-23	TTCCCATCTGTAAGGATCAGG	GGTGACAGCTCTCTGGTGC	70° → 60 (-1°/cycle), then 60°	794
24-25	CTGTCATCATATTCTGCCAGC	GCCACGTCATCTATTCTGG	70° → 60 (-1°/cycle), then 60°	519
26-27	CTTACCCATCAGATTCCTTCG	CACGCCAGTCTTCCACTCG	70° → 60 (-1°/cycle), then 60°	680
28-29	GCAGATGGGAGAGAGGTGC	GGACAGTAGTCAACGCTTCC	70° → 60 (-1°/cycle), then 60°	687

Tabela 3.2: *Primers* adicionais internos e as seqüências utilizadas nas reações

2seqF	TATGGGACCCTGACGATAGG	2seqR	GGCTGGAGTAGGAAGTTGG	4seqF	CCCATAAAGGCTGACTCTGG
4seqR	GGTACTCTCGAGCTCTGG	7seqF	TGGTTGCCTTCCAGTCACC	7seqR	GGAGCTCCCCACTTTTCAGC
10seqF	AGGTGAGTGCCATAAGGTGG	11seqF	CAGACTCTAAGGGACATGG	11seqR	TCATGTCACTGCCTCCAGC
14seqF	GAAACCGGGTAAGCCTTGG	14seqR	GGATTACAATCTCATTTCAGAGG	18seqF	AGACTCAGCATTGGGACC
18seqR	GTGGAGGACGCTGACTGG	20seqF	AGGCAGCCCAGCAACTGAC	20seqR	GGCAGACCTGGATCAGAGG
21seqF	GCTAAGCTGAGCTTCTCTGG	21seqR	CCACGGTGTAGCTTGCAGC	22seqR	AGCGAGCCGTGAGGAAGG
24seqR	GAAGTGTCAGGGAACAGC	27seqF	AGCGCTGGTCTGGGACG	27seqR	CACGGTGTCTCGTAGTTGG
29seqF	GAGGAACATCACTGACACC	29seqR	GGCAGGGAGGGTGTGACG		

Locais onde foi desenvolvido o estudo

1. Ambulatório do Grupo de Estudo de Distúrbios do Movimento da Clínica Neurológica do Hospital das Clínicas da FMUSP.
2. Laboratório de Genética da Erasmus University, Rotterdam.

RESULTADOS

RESULTADOS

Foram investigados 53 probandos com parkinsonismo de início precoce (instalação dos sintomas até 40 anos) ou com história familiar positiva.

Dessa amostra, 29 eram casos esporádicos, 16 apresentavam história familiar sugestiva de padrão de herança AD e 8 com história familiar sugestiva de padrão AR.

No total, 100 amostras de DNA foram colhidas, 70 de pacientes com quadro sugestivo de PP, uma de paciente com parkinsonismo secundário ao uso de neurolépticos e o restante de familiares que não expressavam sinais ou sintomas de parkinsonismo.

Dos pacientes com parkinsonismo, 45 eram do sexo masculino e 25 feminino. A idade média de início do quadro foi de 38,3 anos (10 a 72) e a idade média no momento da investigação era de 49,8 anos (22 a 72). Todos os pacientes tiveram instalação assimétrica dos sintomas e em três (PDBR05.0, PDBR47.0, PDBR01.162) o quadro foi inaugurado por distonia focal.

Quarenta e nove pacientes utilizavam levodopa com boa reposta e destes 27 (55%) desenvolveram discinesias com média de uso do fármaco de 142,9 meses (0,5 a 240 meses) ou 11,9 anos e a dosagem média era de 571,9mg/ dia (187,5 a 2250 mg/ dia).

Os pacientes com padrão de herança AD foram testados para a mutação Gli2019Ser, que é o defeito mais comum do gene *LRRK2*. A investigação de outras mutações neste gene está em andamento.

Os casos esporádicos e com padrão de transmissão AR foram testados para mutações do gene *PARK2*. A investigação mutacional dos genes *PARK6* e *PARK7* está em andamento.

Dos 29 casos de herança AD, a mutação 6099G>A (Gli2019Ser) do gene *LRRK2* foi encontrada em dois pacientes, a probanda PDBR24.0 e o probando PDBR30.0, ambos portadores heterozigotos.

Mutações do gene *PARK2* foram encontradas em 4 famílias com padrão de transmissão AR, todas portadoras homozigóticas, e são:

1. Nova mutação: IVS1+1G>T (PDBR01)
2. c.255delA (PDBR05.0)
3. Deleção de exons 3-4 (PDBR43.0)
4. Deleção de exons 2-3 (PDBR49.0)

Em um probando (PDBR09.0) foi encontrada uma nova mutação G1510C (Gli504Arg) do gene *ATP13A2* (*PARK9*). Apesar de não haver histórico de consangüinidade nos pais, o paciente era homozigoto para a mutação.

Família PDBR24 (*LRRK2*: Gli2019Ser)

PDBR24.0, sexo feminino, 62 anos de idade, iniciou os sintomas de parkinsonismo aos 46 anos com rigidez e tremor no membro superior esquerdo. O curso da doença foi lento e gradual. Há 10 anos foi submetida à

talamotomia no hemisfério direito por não tolerar doses altas de levodopa. Um ano após o procedimento, foi encaminhada para o nosso Ambulatório. Os exames laboratoriais e a ressonância magnética nuclear do encéfalo não apresentam alterações dignas de nota. Não apresenta alterações comportamentais e tampouco cognitivas.

Em 1997, após um ano de uso regular de levodopa desenvolveu leve discinesia global e distonia focal, caracterizada por hiperextensão do hálux. Ambos sintomas melhoraram com ajuste medicamentoso. A paciente faz uso atualmente de levodopa 500 mg/dia, bromocriptina 10 mg/dia e triexifenidila 4 mg/dia com bom controle dos sintomas parkinsonianos. O escore na escala UPDRS, bloco motor, com discinesia na fase *on* é de 22. O escore na escala H&Y na fase *on* é de 2,5.

Os pais da paciente eram primos em primeiro grau. Duas tias, irmãs da mãe, e a mãe apresentavam parkinsonismo, mas não foram avaliadas. O heredograma da família está ilustrado a seguir (Figura 4.1):

Figura 4.1: Heredograma da família PDBR24

PDBR31.0 (*LRRK2*: Gli2019Ser)

O probando PDBR31.0 é neto de portugueses. Seus pais nasceram no Brasil e não eram aparentados. Sempre morou na cidade de São Paulo e era saudável até os 39 anos de idade quando notou tremor em membro superior direito. Posteriormente os sintomas estenderam-se por todo o corpo. Inicialmente foi tratado com levodopa e biperideno com boa resposta e atualmente faz uso de pramipexole 4 mg/dia, amantadina 300mg/dia, tolcapone 200 mg/dia e levodopa 562,5 mg/dia porque doses maiores geram discinesias acentuadas, que se desenvolveram há cerca de dois anos.

A outra familiar afetada é a irmã da mãe que já não deambula mais e faz tratamento em outro serviço e portanto, não pudemos avaliar o caso até o presente momento (Figura 4.2).

O paciente não apresenta nenhuma alteração cognitiva ou comportamental (Mini Exame do Estado Mental: 30/30) e o escore na escala UPDRS, bloco motor em estado *on* é de 18 e o estágio H&Y é de 2,5.

Figura 4.2: Heredograma da família PDBR31

PDBR01 (*PARK2*: IVS1+1G/T)

A família PDBR01 é caucasóide de origem portuguesa. Em 1860 dois irmãos e respectivas famílias migraram para a região noroeste do estado de Paraíba. Devido ao isolamento geográfico e tradição familiar, realizavam casamentos consangüíneos e essa prática perdura até os dias atuais (Figura 4.3). A maior parte dos familiares ainda permanece na Paraíba, mas muitos emigraram para Bahia, Paraná, Brasília e São Paulo.

O probando PDBR01.96 iniciou os sintomas parkinsonianos aos 14 anos e o diagnóstico de PP juvenil foi feito aos 16 anos de idade. Desde os 19 anos é acompanhado no Ambulatório do Grupo de Estudo de Distúrbios do Movimento da Clínica Neurológica do Hospital das Clínicas da FMUSP. Apesar do relato de consangüinidade, a história familiar só pôde ser detalhada com minúcia recentemente.

Em setembro de 2003 foi possível estabelecer a relação de 255 indivíduos a partir dos dados fornecidos pelos familiares que residem no vilarejo na Paraíba. Até Maio de 2004, 26 amostras de DNA de pacientes e familiares foram obtidas. O diagnóstico de PP foi feito em 10 indivíduos.

Uma paciente (PDBR01.149) apresentava parkinsonismo mas de instalação simétrica e após uso de neuroléptico (haloperidol), que fora introduzido por manifestar sintomas psiquiátricos. A coleta do DNA foi realizada porque na ocasião da visita ao vilarejo os critérios de exclusão do estudo ainda não estavam estabelecidos.

Figura 4.3: Heredograma da família PDBR01

A idade média de idade dos pacientes no momento do exame era de 45,1 anos (30-67), e a média de idade de início dos sintomas 30,8 anos (12-46). Todos manifestaram o quadro antes dos 40 anos exceto o caso PDBR01.95 (46 anos). A paciente que apresentava maior pontuação (100) na escala motora do UPDRS era o caso PDBR01.150 possivelmente pela longa duração da doença (24 anos) e pela intolerância a doses maiores de levodopa (250mg/dia) e agonistas dopaminérgicos por apresentar discinesias incapacitantes (Tabela 4.1).

A análise de haplótipos mostrou que os pacientes eram homozigotos para marcadores ao longo do *locus* do gene *PARK2*. O seqüenciamento do gene revelou uma nova mutação em sítio de processamento de RNA (*splicing*) do intron 1, MS1+1G>T, em todos os 10 pacientes diagnosticados com PD. A paciente com quadro de parkinsonismo induzido por neuroléptico era heterozigota para a mutação.

Dos 15 membros da família que não apresentavam sinais ou sintomas de PP, 2 não apresentavam a mutação e 13 eram portadores heterozigotos. A idade desses familiares heterozigotos variavam de 18 a 82 anos (média 54,2) e apenas quatro indivíduos tinham menos de 46 anos de idade, que foi a idade mais tardia de manifestação dos sintomas dos indivíduos afetados. Após a coleta desses dados mais 4 pacientes passaram a ser acompanhados e a idade de início da doença também foi menor que 46 anos (21anos, 22 anos , 27 anos e 45 anos). Entretanto, ainda não está concluída a análise genética desses últimos casos.

Nos homozigotos não foi possível obter DNAC dos exons 1-3 e 4-6 do gene *PARK2* a partir do RNAm extraído de sangue periférico, ao passo que a amplificação de uma banda de tamanho previsível foi obtida de um portador heterozigoto não afetado da mutação IVS1+1G>T e indivíduos controle (Figura 4.4).

A mutação encontrada nesta família leva a falha no processamento do RNAm do gene *PARK2* por afetar um sítio de processamento no exon1, possivelmente levando à formação de um RNAm longo e aberrante que é rapidamente degradado. A falha na obtenção do DNAC é esperada em pacientes parkinsonianos desta família uma vez que eles não têm a proteína RNAm estável. Os achados desta família foram publicados em março de 2006. (Chien et al., 2006).

Figura 4.4: Mutação IVS+1G>T

Legenda: **a)** haplótipo do loco PARK2 de 5 membros da família. Dois marcadores intragênicos do gene *PARK2* estão marcados em azul. **b-d)** Seqüenciamento de parte do exon 1 do gene *PARK2* ilustrando a mutação IVS1 +1G>T (seta vermelha): b) homozigoto, c) portador heterozigoto, d) seqüência normal. **e-g)** Análise por RT-PCR: e) controles, f, g) *PARK2* (*parkin*), a transcrição leva à ausência seletiva da banda correspondente ao gene nos pacientes. C = controles. Seta vermelha = homozigotos para a mutação. Seta cinza = heterozigoto assintomático.

Tabela 4.1: Dados clínicos dos pacientes da família PDBR01

Paciente	Início (ano)	Duração (ano)	Sexo	Exame	TR	BDC	Dist	Assim	BDC	Rigidez	TR	UPDRS	UPDRS	H&Y	Dose	LDopa	Flutuação motora	Discinesia	Neuroimagem	
					Início	Início	Início	Início	exame	exame	exame	off	on	off	on	(mes)		Ldopa		
01.95	46	7	m	53	+	-	-	+	+	+	+	14	-	2,5	-	625	12	+	+	normal
01.96	14	24	m	38	-	+	-	+	+	+	+	14	14	3	500	192	+	+	normal	
01.104	34	34	m	67	+	?	-	+	+	+	+	44	44	4	250	2400	+	+	Leve atrofia	
01.142	38	7	m	45	+	?	?	+	+	+	+	43	43	3	300	84	+	+	?	
01.150	36	24	f	60	+	?	?	+	+	+	+	100	100	5	250	??	+	+	?	
01.162	30	11	m	42	-	-	+	+	+	+	+	29	29	3	500	72	+	+	?	
01.163	35	4	m	39	+	?	?	+	+	+	+	20	20	2,5	0		-	-	?	
01.165	30	6	f	36	+	?	?	+	+	+	+	29	29	2,5	375	??	+	-	?	
01.193	12	18	f	30	+	-	-	+	+	+	+	20	20	2	500	84	+	+	normal	
01.199	33	8	m	41	+	-	-	+	+	+	+	31	31	3	375	18	-	-	normal	

TR= tremor de repouso; BRC= bradicinesia; Dist= distonia; Assim= assimetria; LDopa= levodopa; m= sexo masculino; f= sexo feminino

PDBR05.0 (*PARK2*: c.255delA)

PDBR05.0, 44 anos, é uma paciente do sexo feminino, que apresentou sintomas parkinsonianos aos 25 anos de idade. Dois irmãos de uma prole de 10 também têm parkinsonismo. A mutação do gene *PARK2* detectada, 255delA, é bastante freqüente na população ibérica e espanhola, no entanto, a paciente não sabe referir a origem dos pais e não existe consangüinidade entre eles (Figura 4.5).

O quadro iniciou-se com distonia no membro superior direito e após alguns meses evoluiu com bradicinesia no hemicorpo esquerdo. O curso da doença é longo e progressivo e a paciente mantém o uso de levodopa 500 mg há 16 anos com bom controle dos sintomas. A paciente apresenta discinesias induzidas por levodopa de intensidade leve a moderada que não interferem nas atividades de vida diária. Não apresenta déficits cognitivos e tampouco alterações comportamentais. Os exames laboratoriais e a ressonância magnética de encéfalo não apresentam alterações dignas de nota. O score na escala UPDRS na fase *on* com discinesia leve é de 13 e o estágio H&Y na fase *on* é de 2,5 e na fase *off* é de 4.

Recentemente o irmão mais novo da paciente, com 37anos de idade, foi encaminhado para o nosso Ambulatório (primeiro atendimento em agosto de 2006). Ele não foi incluído nessa casuística porque iniciou o seguimento após a data limite de inclusão (janeiro de 2006). O paciente apresenta parkinsonismo de início precoce (23 anos), início assimétrico (tremor em membro superior esquerdo), curso lento e progressivo, com boa e prolongada resposta à levodopa, da qual faz uso há 10 anos. Há 3 anos tem

discinesias induzidas por levodopa. O material para diagnóstico molecular foi colhido, mas não temos o resultado até o presente momento. O desempenho cognitivo está preservado (Mini Exame do Estado Mental de 29/30), o escore na escala UPDRS, bloco motor, em fase *on* é de 16 e o escore na H&Y, em estado *on* é de 2,5.

Figura 4.5: Heredograma da família PDBR05

PDBR43.0 (*PARK2*: deleção de exons 3-4)

Paciente do sexo feminino, 42 anos de idade, manifestou os primeiros sintomas aos 20 anos de idade com tremor em membro superior esquerdo. Os pais são primos de primeiro grau e a paciente é a única afetada até o presente momento entre os cinco irmãos (Figura 4.6). A evolução da doença foi lentamente progressiva e obtém bom controle dos sintomas atualmente com pramipexole 1,5 mg/dia e amantadina 300 mg/dia. Não apresenta discinesias e não há histórico de distonia. O desempenho cognitivo é normal e não tem alterações comportamentais. A ressonância magnética realizada

em 1996 não evidencia alterações. O escore na escala UPDRS, bloco motor, na fase *off* é de 29 e o escore na escala H&Y é de 2,5 na fase *off*.

Figura 4.6: Heredograma da família PDBR43

PDBR49.0 (*PARK2*: deleção exons 2-3)

PDBR49.0 é uma paciente do sexo feminino, 48 anos, filha de pais consanguíneos (primos em primeiro grau) e a única afetada até o momento de uma prole de cinco filhos (Figura 4.7). A manifestação inicial foi tremor em membro superior direito aos 25 anos que lentamente progrediu afetando os quatro membros. Atualmente a paciente tem instabilidade postural acentuada com freqüentes episódios de queda e freezing. Utiliza levodopa há 15 anos na dose de 500 mg/ dia. A impossibilidade de uso de altas doses de levodopa e agonistas dopaminérgicos deve-se ao fato de ter discinesias incapacitantes além de ter intolerância a várias drogas antiparkinsonianas. O

escore na escala UPDRS, bloco motor, na fase *on* com discinesia leve é de 23 e o escore na escala H&Y na fase *on* é de 3.

Figura 4.7: Heredograma da família PDBR49

PDBR09.0 (*ATP13A2*/ *PARK9*: Gli504Arg)

O paciente PDBR09.0, 25 anos, masculino, apresenta a mutação Gli504Arg no gene *ATP13A2* em homozigose (Figura 4.10 e Figura 4.11). Ele é o mais jovem de uma prole de quatro, de pais não consanguíneos. A origem étnica dos pais é incerta, mas provavelmente descendem de portugueses que habitavam na região nordeste do país. Nenhum caso similar foi relatado na família (figura 4.8).

O desenvolvimento neuropsicomotor foi normal até os 12 anos de idade quando os familiares, amigos e professores notaram bradicinesia e incoordenação motora. Um ano após a instalação desses sintomas, parkinsonismo juvenil foi diagnosticado e iniciou o uso de agonistas

dopaminérgicos. Pela pobre resposta, levodopa foi associada após algum tempo do diagnóstico.

A evolução da doença foi lenta e gradual com quadro rígido-acinético, pois nunca manifestou tremor de repouso ou alteração esfíncteriana. O desempenho escolar sempre foi satisfatório, obtendo boas notas e terminou o segundo grau sem intercorrências.

Em 2002 o paciente passou a ser acompanhado em nosso Ambulatório. Na ocasião utilizava levodopa e bromocriptina com bom controle dos sintomas e não apresentava alterações comportamentais ou cognitivas evidentes. No entanto, alguns meses após o início do seguimento, desenvolveu discinesias, alucinações visuais e episódios de agressividade. O quadro comportamental melhorou após diminuição da dose diária da levodopa e introdução de quetiapina.

O exame físico evidencia quadro rígido-acinético, reflexos miotáticos exaltados, porém preservação do reflexo cutâneo-abdominal e resposta normal do reflexo cutâneo-plantar, ausência de espasticidade e presença de paralisia supranuclear do olhar vertical para cima.

Nos últimos anos as opções terapêuticas ficaram restritas e atualmente, para evitar discinesias intensas, utiliza baixas doses de levodopa (562,5 mg/dia) e mantém o uso de quetiapina na dose de 50 mg/dia. Nesse regime realiza as atividades de vida diária sem requerer grandes auxílios. O escore na escala H&Y em fase *on* é de 4 e o escore no bloco motor da escala UPDRS fase *on* é de 37 com leves discinesias no pico de dose da levodopa.

Apesar do quadro comportamental o escore no Mini Exame do Estado Mental é de 30 e está atualizado sobre eventos políticos e sociais atuais. O teste neuropsicológico está sendo realizado e por requerer várias sessões o resultado final ainda não está concluído.

A tomografia computadorizada do crânio de 2006 mostra atrofia moderada e difusa nos hemisférios cerebrais e cerebello (vide Figura 4.9 a seguir).

Figura 4.8: Heredograma da família PDBR09

Figura 4.9: Tomografia computadorizada do crânio do paciente PDBR09.0

Figura 4.10: Seqüenciamento genético mostrando a mutação Gli504Arg

A: Seqüenciamento de parte da seqüência genômica do gene *ATPA13A2*. A posição da mutação é indicada por uma seta.

B: Conservação evolutiva da proteína ATP13A2 em diferentes espécies destacando o local da mutação Gli504Arg

Figura 4.11: Proteína ATP13A2

Representação esquemática da proteína ATP13A2 e os domínios de função. A mutação G504R (Gli504Arg) está sublinhada.

DISCUSSÃO

DISCUSSÃO

Acredita-se que apenas 10 a 15% dos casos de PP sejam monogênicos (Bonifati et al., 2004a). Dessa forma, tanto fatores genéticos, como ambientais são preponderantes na etiologia do PP. No entanto, a identificação de várias formas monogênicas de DP com padrão de transmissão Mendeliano tem auxiliado muito na elucidação etiopatogênica desta doença.

A nossa série de pacientes apresenta quadro clínico similares ao da DP idiopática. Os indivíduos relataram início assimétrico de apresentação dos sintomas, a maioria responde bem ao tratamento com agonista dopaminérgico ou levodopa e há o desenvolvimento de discinesia após uso prolongado de levodopa.

A seguir serão abordadas em tópicos específicos as mutações encontradas neste estudo.

5.1 Mutação Gli2019Ser no gene *LRRK2*

Foram encontrados na nossa série dois casos de heterozigotos para a mutação Gli2019Ser no gene *LRRK2* (probandos PDBR24.0 e PDBR31.0). Entretanto, deve-se ressaltar que apenas a Gli2019Ser foi analisada. A pesquisa de outras possíveis mutações do gene *LRRK2* está em andamento. Neste contexto a frequência da mutação Gli2019Ser em nossa

amostra é de 12,5% (2 em 16). No artigo de Di Fonzo et al. (2005) foram investigadas 61 famílias, com padrão AD, sendo nove da nossa amostra. Foram encontradas 4 famílias portadoras dessa mutação, ou seja 6,6%. Esses achados confirmam a associação do gene *LRRK2* com neurodegeneração e identificam uma mutação comum em casos de PD com padrão de herança AD.

De modo similar, estudos conduzidos na Europa e nos Estados Unidos da América evidenciam a mutação Gli2019Ser como a mais comum do gene *LRRK2* e a sua frequência em casos familiares é de 5 a 6% e de 1 a 2% em DP de origem esporádica (Cookson et al., 2005; Goldwurm et al., 2005).

Apesar da frequência da mutação na nossa população ser um pouco maior à descrita na literatura, deve-se levar em consideração que não temos um estudo epidemiológico da frequência da mutação Gli2019Ser entre parkinsonianos no Brasil.

Brice (2005), porém aponta que a frequência dessa mutação pode variar de 3 a 41% dependendo da população de estudo. Na população judaica askenazita norte-americana, Ozelius et al. (2006) encontraram uma frequência de 18,3% (22 dentre 120 casos) da mutação Gli2019Ser em pacientes com DP de origem familiar ou esporádica e 1,3% (4 em 317) no grupo controle. Nas DP familiares, a frequência era maior: 29,7% (11 em 37).

Lesage et al. (2006), em outro estudo, também abordando populações isoladas, encontraram uma frequência de 39% (30 de 76 probandos) da

mutação Gli2019Ser em pacientes árabes do norte da África com DP e 3% em grupo controle (2 de 69 controles). Nesse grupo a frequência da mutação em casos esporádicos e familiares era respectivamente 41% e 37%.

Os dois estudos populacionais acima descritos evidenciam, portanto, que há prevalência maior da mutação em populações específicas. Por outro lado, em países como Itália, Espanha e Portugal além da alta prevalência da mutação Gli2019Ser há indícios de um ancestral comum para a (Paisan-Ruiz et al., 2005; Goldwurm et al., 2005). Esse fato pode explicar em parte a nossa frequência da Gli2019Ser (12,5%) uma vez que os dois heterozigotos encontrados são descendentes de portugueses e este grupo contribui significativamente para a formação de nossa população.

Deve-se ressaltar, contudo, que a frequência dessa mutação é muito baixa em países asiáticos (Fung et al., 2006) e norte da Europa (Bonifati, 2006).

Até o presente momento o gene *LRRK2* é o mais importante determinante de parkinsonismo de causa genética em diversas populações. Há a necessidade de estudar grandes séries de etnias diferentes pareadas para comparar a prevalência das mutações desse gene, principalmente da mutação Gli2019Ser. O esclarecimento da penetrância e expressão gênica das mutações do *LRRK2* poderá explicar o porquê da variabilidade da idade de início dos sintomas e possíveis variações fenotípicas. Outra linha de pesquisa importante deve ser direcionada para determinar os fatores ambientais ou genéticos que podem influenciar a manifestação da doença e a sua progressão já que recentemente padrões digênicos (*LRRK2* e *PARK2*)

foram encontrados em parkinsonismos familiares (Paisan-Ruiz et al., 2005; Lesage et al., 2006).

5.2 Mutações no gene *PARK2*

Das oito famílias com padrão compatível com herança AR e início precoce das manifestações, mutações do gene *PARK2* foram encontradas em quatro famílias, ou seja, 50% de frequência, que é a mesma descrita para análise de grande série de parkinsonismo de início precoce com padrão de herança AR (Lucking et al., 2000).

Em uma das famílias, PDBR01, foi encontrada uma nova mutação (IVS1+1G>T) em sítio de processamento (*splicing*) no gene *PARK2* (Chien et al., 2006). A característica primordial dessa família está na preponderância de casamentos consangüíneos durante várias gerações.

Uma extensa revisão publicada recentemente listou 95 mutações encontradas no gene *PARK2* (Hedrich et al., 2004) e nessa série apenas 4 mutações estavam localizadas em sítios de processamento. No estudo de Bertoli-Avella et al. (2005) também foi descrita uma nova mutação em sítio de processamento de RNA, a IVS11-3C>G, num paciente cubano filho de pais consangüíneos.

A mutação IVS1+1G/T possivelmente resulta na falha de processamento do RNAm do gene *PARK2* levando à formação de um RNAm aberrante e longo que por ser instável poderia ser rapidamente degradado. Desta forma a ausência de RNAm normal observado nos pacientes homozigotos para a mutação por meio da análise RT-PCR já era

esperada. Da mesma maneira, a obtenção de RNAm normal em portadores heterozigotos reforça a hipótese acima.

Na família PDBR01 observa-se uma co-segregação completa da mutação em estado homozigótico com a expressão da doença e ausência de parkinsonismo em portadores heterozigotos. Esse dado reforça a perda da função protéica comum em doenças de herança AR. Observa-se também que nesta família não há o fenômeno de haploinsuficiência uma vez que os portadores heterozigotos da mutação do gene *PARK2* não tiveram maior susceptibilidade à DP. A exceção é o caso PDBR01.210 em que apresentou um quadro de parkinsonismo de instalação simétrica e que se manifestou após uso de neuroléptico (haloperidol). Infelizmente a chance de suspensão do neuroléptico é remota e a troca do haloperidol por neurolépticos que minimizam a indução de parkinsonismo também não é possível.

A observação de que nessa família os portadores heterozigotos não manifestaram parkinsonismo não exclui a possibilidade de que outras mutações do gene *PARK2* em heterozigose não possam desenvolver ou aumentar a susceptibilidade para DP.

De fato, Cookson et al. (2003) demonstraram que nem todas as mutações do gene *PARK2* têm mecanismos fisiopatológicos similares. As mutações podem resultar em perda de função típica de padrão AR ou levar à haploinsuficiência em alguns padrões AD, ou em ganho de função, comum em doenças AD. Esta última característica é observada em algumas mutações do gene *PARK2* como Arg256Cis e Arg275Trp, localizadas no terminal RING 1 da proteína parkina, gerando inclusões citoplasmáticas e

nucleares que resultam na formação de agressomas. Os agressomas são inclusões proteináceas formadas no centrossomo em resposta ao estresse proteolítico. Eles seqüestram proteínas defeituosas ou desnecessárias (Olanow et al., 2004). Em circunstâncias normais, os agressomas são degradados pelo sistema proteassomal, mas nessas mutações, o acúmulo não é eliminado pelo sistema ubiquitina-proteassoma, pois a parkina que é uma E3 ubiquitina ligase não é funcional. Na revisão de Hendrich et al. (2004), os autores relataram casos de parkinsonismo em heterozigotos para a mutação Arg275Trp.

Um ponto importante na família PDBR01 é que além do parkinsonismo, ela oferece também oportunidade para pesquisar e estudar outras doenças genéticas. Durante a investigação descobrimos casos de amiotrofia muscular progressiva, -talassemia e distúrbio visual precoce resultando em amaurose na fase adulta.

Na revisão de Hedrich et al. (2004), os autores constataram as mutações mais comuns do gene *PARK2* em ordem de frequência eram: deleção do exon 4, deleção do exon 3, mutação de uma base no exon 7 (C924T) e deleção simples de base no exon 2 (del255 ou 256A). Essas cinco mutações são responsáveis por 35% de todas as mutações do gene *PARK2*.

Encontramos uma paciente homozigota para a mutação del255A, mas sem histórico de consangüinidade na família. Acreditava-se que essa deleção ocorria mais frequentemente na população ibérica ou espanhola

(Muñoz et al., 2002), porém Hedrich et al. (2004) constataram que ela ocorria em diferentes grupos étnicos.

Duas pacientes apresentavam respectivamente deleção de exons 3-4 (PDBR43.0) e deleção de exons 2-3 (PDBR49.0) no gene *PARK2*. Conforme acima referido, as deleções nos exons 3 e 4 são extremamente freqüentes. Dessa forma, excetuando a família PDBR01, as mutações do gene *PARK2* que foram encontradas nesse estudo estão entre as mais comuns descritas na literatura.

As mutações del255A e deleção do exon 3 e 4 do gene *PARK2* foram previamente descritas no Brasil em um paciente parkinsoniano, heterozigoto composto para ambas as mutações. Este caso foi incluído em estudo multicêntrico conduzido por Rawal et al. (2001), que teve a participação do Grupo de Estudo de Distúrbios do Movimento do Hospital das Clínicas da Faculdade de Medicina da Universidade Federal do Paraná.

5.3 Mutação Gli504Arg no gene *ATP13A2*

Recentemente foi descrita uma família chilena com parkinsonismo juvenil com predomínio do quadro rígido-acinético associado a, espasticidade, paralisia do olhar vertical e demência. Esse quadro clínico era semelhante ao dos casos da família de Kufor-Rakeb. O estudo genético encontrou ligação entre as duas famílias na mesma região cromossômica e permitiu com que os autores identificassem uma mutação no gene *ATP13A2*. Os afetados da família jordaniana de Kufor-Rakeb eram portadores homozigotos de uma duplicação de 22 nucleotídeos no exon 16.

(1632_1653dup22) que introduz uma alteração no quadro de leitura (*frameshift*) do RNAm. Os pacientes da família chilena eram heterozigotos composto das seguintes mutações: deleção de um nucleotídeo no exon 26 (3057delC) que resulta em uma parada do quadro de leitura e código de parada prematura (1019GfsX1021) e no outro alelo, uma mutação no sítio de processamento no exon 13 (IVS13+5G>A) levando a ausência desse exon no transcrito e na conseqüente ausência de 111 aminoácidos na proteína (Ramirez et al., 2006).

Encontramos um caso com a mutação no gene *ATP13A2* em nossa amostra em homozigose, apesar de não haver relato de consangüinidade entre os pais. A mutação G1510C no exon 15 leva a uma substituição simples de aminoácido, Gli504Arg. O aminoácido Gli504 é altamente conservado entre os mamíferos e está localizado na grande alça da porção citosólica da proteína ATP13A2, num sítio de provável fosforilação catalítica. A introdução do aminoácido arginina de carga positiva no lugar de um aminoácido pequeno e de carga neutra, glicina, provavelmente resulta na perda da função protéica.

Acredita-se que a proteína ATP13A2 seja uma translocase lisossomal (Ramirez et al., 2006) e como os lisossomos são importantes para a degradação de α -sinucleína (Cuervo et al., 2004) a disfunção destas organelas pode resultar no acúmulo da α -sinucleína e formação de CL.

Há uma discussão na literatura sobre a possibilidade da síndrome de Kufor-Rakeb ser ou não considerada uma de PP, pois o fenótipo dos pacientes inclui manifestações neurológicas outras além do quadro

parkinsoniano que são: sinais de lesão piramidal, paralisia do olhar vertical, mioclonias face-fauce-dedos, déficit cognitivo importante. Além desse quadro clínico atípico a evolução geralmente é mais rápida que as demais formas de PP (Williams et al., 2005).

O quadro clínico do paciente PDBR09.0, conforme descrito anteriormente (vide resultados) apresenta algumas diferenças em relação aos casos da síndrome de Kufor-Rakeb já relatados na literatura. Assim, neste caso, no quadro neurológico não havia síndrome piramidal franca, embora hiperreflexia estivesse presente; o desempenho cognitivo era satisfatório; não se constatou a presença de mioclonias de face-fauce-dedos; e a evolução foi muito mais lenta comparada com a forma já conhecida da doença. Além dessas diferenças a resposta à levodopa foi melhor do que a esperada nesta condição. As complicações motoras e psiquiátricas surgiram após vários anos de uso dos medicamentos antiparkinsonianos.

Este caso nos leva a considerar que o fenótipo por mutações no gene *ATP13A2* é variável e o tipo da mutação deve contribuir para a diversidade do quadro clínico. Os achados genéticos das famílias jordanianas e chilenas revelam mutações levando a proteínas truncadas enquanto que no presente caso a mutação é pontual, levando a uma substituição simples de aminoácido. Este fato pode contribuir para uma expressão fenotípica mais leve da doença.

Outro ponto a ser considerado é o fato de que foram encontradas mutações em dois heterozigotos italianos (Tre12Met e Gli533Arg) por

Bonifati et al., (comunicação pessoal) que manifestavam parkinsonismo juvenil sem outras expressões fenotípicas. A interpretação para a existência de heterozigotos sintomáticos é difícil por se tratar de uma doença de herança AR. Uma das possibilidades é que a mutação no gene *ATP13A2* mesmo em heterozigose é um fator de predisposição para desenvolvimento da DP, embora a presença de uma mutação não identificada no outro alelo também deva ser considerada.

A elucidação da causa da variabilidade fenotípica só poderá ser esclarecida quando mais casos de mutações no gene *ATP13A2* forem encontrados e as manifestações clínicas forem descritas. Outro fato a ressaltar é que a investigação desse gene é importante e não deve ser negligenciada principalmente nos casos de parkinsonismo juvenil sem outras manifestações neurológicas uma vez que o fenótipo das mutações do gene *ATP13A2* pode ser extremamente variável.

5.4 Considerações Finais

Os desafios a enfrentar no estudo da genética da DP são muitos e incluem: 1) definir o espectro genético e clínico das formas monogênicas; 2) estabelecer a terminologia e classificação da síndromes parkinsonianas frente às descobertas no campo genético; 3) identificar os fatores de susceptibilidade genética; 4) desenvolver condutas para o teste genético na DP; 5) pesquisar os mecanismos de degeneração neuronal e as compensações funcionais em modelos genéticos experimentais para melhor

elucidação da patogênese, o que auxiliará no desenvolvimento de novos fármacos para terapia clínica e neuroproteção (Klein, 2006a).

A dificuldade de estudar as formas monogênicas da DP recai na sua baixa incidência. Além disso, a relação genótipo-fenótipo nem sempre é uniforme. Algumas vezes a presença de uma mutação em heterozigose em doença de padrão AR pode atuar como um fator de susceptibilidade à doença e resulta no fenômeno dominante-negativo.

O termo DP idiopática refere-se ao parkinsonismo de início tardio, sem indícios de hereditariedade e cuja autópsia evidencia perda neuronal com gliose de astrócitos e formação de inclusões intracitoplasmáticas típicas cerebrais chamadas de corpúsculos de Lewy. As descrições de casos de parkinsonismo de etiologia genética definida, mas com quadro clínico e anátomo-patológico indistinguíveis da DP idiopática gera controvérsias quanto ao termo adequado para denominá-los. Dentre as formas monogênicas de parkinsonismo, a síndrome de Kufor-Rakeb é a que merece maiores discussões uma vez que apresenta atípias marcantes.

Uma das dificuldades para o estudo da genética dos PP é que não há um teste específico para o diagnóstico de DP, pois o diagnóstico é clínico. O diagnóstico diferencial com outras síndromes parkinsonianas muitas vezes é difícil e confirmado apenas com estudo anátomo-patológico.

Quanto aos genes envolvidos na susceptibilidade para o desenvolvimento da DP, Pankratz et al. (2003) demonstraram por meio de estudo de ligação que os cromossomos 2, 10 e X devem ser considerados. Em 2005, Maraganore et al., realizaram um estudo de associação em que

comparam a frequência de polimorfismos em todo o genoma e indicam que alguns deles localizados nos genes *SEMA5A*, *PARK10* e *PARK11* aumentam o risco para desenvolvimento da DP. Outras mutações presentes nos genes *NAT2* (N-acetiltransferase 2), *MAOB* (monoamino oxidase B) (Klein e Schlossmacher, 2006b) e mutações no gene *GBA* (beta-glucosidase ácida) causadora da doença de Gaucher também podem aumentar o risco de desenvolvimento de parkinsonismo (Lwin et al., 2004; Spitz et al., 2005). Porém pouco sabemos ainda dos mecanismos da susceptibilidade e interação com fatores ambientais que podem modificar o curso da doença, idade de início, manifestação clínica e duração.

Quanto à questão dos testes genéticos alguns pontos relevantes devem ser discutidos. O primeiro é quanto ao propósito do teste genético. Na prática clínica o teste genético visa identificar o indivíduo portador de determinada mutação para fins de intervenção preventiva (fase pré-sintomática) ou clínica (sintomática) que podem mudar o curso da doença. No caso da DP a identificação precoce não auxilia a prevenção por ainda não haver terapia neuroprotetora ou terapia gênica e o diagnóstico genético na fase sintomática não muda a estratégia terapêutica.

Mesmo que os testes sejam disponíveis comercialmente estes não devem ser realizados sem que haja a presença de uma equipe multidisciplinar para o aconselhamento genético visando orientar e esclarecer as implicações do teste e as condutas a serem tomadas caso venha a ser positivo, principalmente nas situações em que a penetrância do gene é variável.

Os casos de parkinsonismo por mutações genéticas, descritos na literatura, apresentam ampla heterogeneidade clínica e genética. Embora os testes de DNA possam no futuro indicar condutas preventivas ou terapêuticas, recomenda-se que atualmente sejam restritos para fins de pesquisa científica (McInerney-Leo, 2005; Klein e Schlomossmacher, 2006b; Tan e Jankovic, 2006).

Desde a descrição do primeiro gene envolvido na gênese do parkinsonismo em 1997, novas descobertas sobre a fisiopatologia da DP ocorreram. A contribuição da genética é vital e estudos futuros devem ser estimulados. Espera-se que com os novos conhecimentos, avanços na terapêutica possam suceder, principalmente nos campos da neuroproteção, terapia gênica, prevenção e intervenção para mudanças do curso da doença.

CONCLUSÕES

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4. Encontramos mutações dos genes *PARK2* e *LRRK2* que são até o presente momento as mais freqüentes na formas familiares de parkinsonismo de herança autossômica recessiva e dominante, respectivamente.
5. As seguintes mutações do gene *PARK2* foram encontradas em 4 famílias (todos os indivíduos eram portadores homozigóticos: a) IVS1+1G>T (família PDBR01); b) 255delA (família PDBR05); c) deleção de exons 3-4 (família PDBR43); d) deleção de exons 2-3 (família PDBR49).
6. A mutação Gli2019Ser do gene *LRRK2* foi encontrada nos probandos PDBR24.0 e PDBR31.0.
7. Os padrões de apresentação clínica dos indivíduos afetados por mutações dos genes *PARK2* e *LRRK2* eram semelhantes aos descritos na literatura.
8. Foi encontrada uma nova mutação em homozigose no gene *ATP13A2* levando a uma substituição simples de aminoácido Gli504Arg no probando PDBR09.0.
9. Os achados clínicos do paciente PDBR09 diferem em alguns aspectos dos descritos na literatura.

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ARTIGOS PUBLICADOS

Novel *parkin* Mutations Detected in Patients With Early-Onset Parkinson's Disease

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Abstract: A multiethnic series of patients with early-onset Parkinson's disease (EOP) was studied to assess the frequency and nature of *parkin*/PARK2 gene mutations and to investigate phenotype–genotype relationships. Forty-six EOP probands

with an onset age of <45 years, and 14 affected relatives were ascertained from Italy, Brazil, Cuba, and Turkey. The genetic screening included direct sequencing and exon dosage using a new, cost-effective, real-time polymerase chain reaction method. Mutations were found in 33% of the indexes overall, and in 53% of those with family history compatible with autosomal recessive inheritance. Fifteen *parkin* alterations (10 exon deletions and five point mutations) were identified, including four novel mutations: Arg402Cys, Cys418Arg, IVS11-3C>G, and exon 8-9-10 deletion. Homozygous mutations, two heterozygous mutations, and a single heterozygous mutation were found in 8, 6, and 1 patient, respectively. Heterozygous exon deletions represented 28% of the mutant alleles. The patients with *parkin* mutations showed significantly earlier

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Received 5 March 2004; Revised 28 April 2004; Accepted 3 July 2004

Published online 6 December 2004 in Wiley InterScience (www.interscience.wiley.com). DOI: 10.1002/mds.20343

onset, longer disease duration, more frequently symmetric onset, and slower disease progression than the patients without mutations, in agreement with previous studies. This study confirms the frequent involvement of *parkin* and the importance of

genetic testing in the diagnostic work-up of EOP. © 2004 Movement Disorder Society

Key words: Parkinson's disease; early-onset; parkin; gene dosage; mutation

Autosomal recessive forms are increasingly recognized among patients with early-onset Parkinson's disease (EOP),¹ and mutations in three genes, *parkin*,² *DJ-1*,³ and *PINK1*,⁴ have been identified. *Parkin* mutations vary from point mutations to complex rearrangements, including deletions and/or multiplications of complete exons.^{5–10} Gene copy dosage assays are, therefore, important in the mutational analysis of *parkin*, but the reported frequency of exon rearrangements varies greatly (33 to 67%).^{6–10} Most *parkin* mutations lead to the loss of the ubiquitin E3 ligase activity of the encoded protein, which normally tags specific substrates for degradation through the ubiquitin–proteasome pathway.¹¹ However, the mechanisms by which *parkin* mutations cause neurodegeneration remain to be elucidated.

Patients with *parkin* mutations are difficult to distinguish from other forms of EOP on the basis of the clinical features.^{6,12} Moreover, due to the complexity of the *parkin* gene and the wide spectrum of mutations, the genotype–phenotype correlations are poorly understood. There is a wide variation in the clinical presentation and age at onset, even in patients with the same mutation.¹² Atypical clinic and genetic presentations, including pseudodominant inheritance^{13,14} have also been described. Last, in a few patients, only one heterozygous mutation is detected, suggesting that a second mutation still escapes detection by current screening methods or that some mutations in heterozygous form are sufficient to cause this disease.^{8,15,16} It is clear that much work is still ahead to disentangle the complexity of the disease associated with *parkin* mutation (the “parkin disease”), and the analysis of further, large series of patients is warranted.

Here, we report on the nature and frequency of *parkin* mutations and on phenotype–genotype relationships in a newly ascertained, multiethnic group of EOP patients. Genetic screening included direct sequencing of the *parkin* coding region and a novel, cost-effective quantitative polymerase chain reaction (PCR) method for exon dosage analysis.

PATIENTS AND METHODS

Patients

We included in the study all the patients referred from the participating centers during the period 2000 to 2002,

who fulfill the following criteria: clinical diagnosis of Parkinson's disease (PD), and either (1) positive family history compatible with autosomal recessive inheritance and age at onset ≤ 45 years in the index case, or (2) isolated presentation with age at onset ≤ 40 years. According to these criteria, we collected a multiethnic group of 46 EOP index patients from Italy ($n = 39$), Brazil ($n = 4$), Cuba ($n = 2$), and Turkey ($n = 1$), plus 14 affected first-degree relatives (total sample set $n = 60$). There were 17 index cases from families compatible with autosomal recessive inheritance, and 29 were isolated patients. Consanguinity was reported in eight families and two isolated cases.

The clinical diagnosis of Parkinson's disease was established when at least two of the three cardinal signs (resting tremor, rigidity, and bradykinesia) and a positive response to dopaminergic therapy were present, in absence of atypical features or other causes of parkinsonism, according to the UK Parkinson's Disease Society Brain Bank criteria.^{17,18} Neurological examination was performed by neurologists with experience in movement disorders and included the Unified Parkinson's Disease Rating Scale (UPDRS, Motor part)¹⁹ and Hoehn and Yahr scale²⁰ in *on* and (if possible) in *off* status. Clinical data were collected using a standard form. Informed consent was obtained from all patients. Venous whole blood was taken and DNA isolated according to standard procedures.²¹

Molecular Studies

Haplotype Analysis.

In the families compatible with autosomal recessive inheritance, we typed short tandem repeat (STR) markers from the *PARK2/parkin*,² *PARK6/PINK1*,⁴ and *PARK7/DJ-1*³ regions, using PCR with fluorescently labeled primers and an ABI 3100 automatic DNA analyzer, as detailed previously.²² Haplotypes were constructed based on the minimum number of recombinations.

Screening of Homozygous Deletions and Direct Sequencing.

Families showing no sharing for both haplotypes (homozygous or heterozygous) at the *PARK2* locus were excluded from the mutational screening ($n = 3$). For the remaining families and the isolated patients, all 12 exons

and exon–intron boundaries of the *parkin* gene were amplified using intronic primers as described.²³ For exons 1, 6, and 10, we designed new intronic primers (primers and PCR conditions available on request). Homozygous exon deletions were identified by agarose gel analysis,^{2,5} and the patients concerned were excluded from further screening. Direct sequencing of the *parkin* gene was performed using the BigDye terminator chemistry (Applied Biosystems). PCR products were loaded on an ABI 3100 Automatic DNA sequencer and analyzed with the SeqScape software version 1.1 (Applied Biosystems). The frequency of the novel detected variants was assessed in panels of at least 96 and up to 500 chromosomes from ethnically matched control individuals, by digestion with restriction enzymes or by the allele specific oligonucleotide technique. We used five computer programs to predict the possible consequences on splicing of sequence changes in the proximity of the exon–intron boundaries.^{24–28}

Exon Dosage Analysis.

All index patients with a single heterozygous mutation or no mutations detected by previous analyses were further investigated for heterozygous exon rearrangements. Exon dosage was performed through quantitative PCR using an iCycler iQ Real-time PCR machine (BioRad) and SYBR Green I as intercalation dye.

Exonic and intronic primers for the 12 exons of the *parkin* gene were designed (available on request), allowing amplification of genomic fragments ranging from 81 to 139 bp. Fifty nanograms of genomic DNA were used as template to perform single PCR reactions (final volume, 25 μ l, qPCR Core kit, Eurogentec) for *parkin* and a “control gene” (*β -globin*, *HBB*); all samples were tested in triplicate, and at least one positive and two negative controls were included in every plate (96-well plates). The thermal cycling parameters were as follows: 95°C, 10 minutes, 40 cycles of 95°C, 20 seconds, 60°C, 45 seconds, 75°C, 15 seconds, enabling for real-time data collection. A melting curve was generated for each sample, allowing the detection of nonspecific products during the amplification.

The fluorescence of the SYBR Green increases significantly as it binds and intercalates into double-stranded DNA during the extension step of the amplification cycle. At some point during amplification, the accumulation of product results in a measurable change in fluorescence of the reaction mixture; this point is called the threshold cycle (CT). We used this value to perform our calculations, given that there is a linear relationship between the log of the starting amount of template and the corresponding CT during real-time PCR.²⁹

The *iCycler* software (v. 3.0a) calculates automatically the CT for every well. Because three different measurements are obtained per sample, the average CT and standard deviation (SD) are calculated for both *parkin* and *β -globin*. The average CT was used to calculate the ratio *parkin*/ *β -globin* ($R_{P/\beta}$) using the following formula:

$$R_{P/\beta} = [(CCT_{Parkin} - CCT_{\beta globin}) - (PCT_{Parkin} - PCT_{\beta globin})]^2$$

where CCT is the average CT for the negative (normal) control sample and PCT is the average CT for the patient sample. On the basis of the observed variability of the values of the ratios in normal individuals and positive controls with *parkin* heterozygous rearrangements, we considered as normal the ratios between 0.8 and 1.2. Values lower than 0.7 or higher than 1.3 are interpreted as heterozygous deletion or duplication of the assessed exon, respectively. All positive results were confirmed at least twice, and an average ratio was calculated. Furthermore, all cases with homozygous or heterozygous exon deletions affecting only one exon were confirmed with an independent set of primers to avoid false-positive results due to primer mismatch caused by undetected polymorphisms. Segregation of detected rearrangements was tested whenever DNA samples from relatives were available. The consequences of the exon deletions on the protein (in-frame or frameshift) were estimated based on the *parkin* cDNA sequence published with accession no. AB009973.

Statistical Analysis

All calculations were done using *SPSS v. 11* software (SPSS, Chicago, IL). We used the nonparametric Mann–Whitney *U* test or the Student’s *t* test for comparison of means and the χ^2 or Fisher’s exact test for comparison of proportions when appropriated. Differences of means (disease severity, UPDRS and Hoehn and Yahr score in *off*) were tested using analysis of covariance. *P* values for trends were obtained from simple linear regression models, where type of mutation was included as a continuous term (0, no *parkin* mutation; 1, two *parkin* exon deletions; 2, *parkin* heterozygous point mutation).

RESULTS

Clinical Studies

Patient characteristics are summarized in Table 1. The mean age at onset (AAO) was 33 ± 11 years, ranging from 14 to 65 years. Resting tremor and bradykinesia at

TABLE 1.
Phenotype description of the complete sample set and according to parkin genotype

Characteristics	Total sample set	n	Patients with <i>parkin</i> mutations	n	Patients without <i>parkin</i> mutations	n
Gender male (%)	34 (57)	60	11 (48)	23	23 (61)	37
Age at onset, yr (range)	33 ± 11 (14–65)	60	28 ± 9 (15–44) ^a	23	39 ± 10 (14–65)	37
Disease duration, yr (range)	15 ± 9 (1–36)	59	20 ± 9 (6–36) ^b	22	13 ± 8 (1–30)	37
Age at examination, yr (range)	49 ± 10 (19–71)	59	49 ± 10 (32–70)	22	49 ± 10 (19–71)	37
Symptoms and signs at onset						
Bradykinesia (%)	31 (54)	57	10 (48)	21	21 (58)	36
Resting tremor (%)	30 (53)	57	10 (48)	21	20 (56)	36
Asymmetry (%)	46 (79)	58	13 (62) ^a	21	32 (89)	37
Dystonia (%)	7 (12)	57	3 (14)	21	4 (11)	36
Clinical signs at examination						
Bradykinesia (%)	55 (96)	57	21 (100)	21	34 (94)	36
Resting tremor (%)	37 (66)	56	14 (67)	21	23 (66)	35
Rigidity (%)	52 (91)	57	19 (90)	21	33 (92)	36
UPDRS off (range)	49 ± 21.2 (6–90)	24	41 ± 20.5 (6–70) ^c	8	53 ± 22 (18–90)	16
UPDRS on (range)	20 ± 11.0 (2–45)	42	20 ± 12.8 (2–43)	14	21 ± 10.1 (1–45)	28
Hoehn & Yahr off (range)	3.3 ± 0.9 (1–5)	30	2.9 ± 0.9 (1–4) ^d	10	3.4 ± 0.9 (2–5)	20
Hoehn & Yahr on (range)	1.8 ± 0.7 (0–4)	48	1.9 ± 0.9 (0–4)	17	1.7 ± 0.6 (1–2.5)	31
Treatment						
With L-dopa (%)	46 (88)	52	17 (81)	21	29 (93)	31
Daily dose of L-dopa, mg (range)	556 ± 304 (100–1,250)	44	497 ± 337 (150–1,250)	15	587 ± 288 (100–1,200)	29
Duration, mo. (range)	123 ± 92 (3–336)	36	139 ± 102 (8–290)	13	115 ± 87 (3–336)	23
Other features (%)						
L-Dopa-induced dyskinesias	34 (79)	45	12 (75)	16	21 (72)	29
L-Dopa-induced motor fluctuations	33 (73)	43	10 (63)	16	24 (89)	27

^a*P* = 0.02; ^b*P* = 0.005.

^c*P* = 0.06; ^d*P* = 0.006 (the last two after adjustment for disease duration).

UPDRS, Unified Parkinson's Disease Rating Scale.

onset were found in around half of the patients (53 and 54%). The onset of signs was asymmetric in most of them (79%).

At examination, bradykinesia and rigidity were the most frequent signs, present in 96% and 91% of the patients, respectively. Additional features at examination included sleep benefit (present in 9 cases), depression (8 cases), psychosis (4 cases), severe anxiety (2 cases), and panic attacks (1 case). A total of 88% of the patients received treatment with levodopa; the vast majority of them also presented L-dopa-induced dyskinesias (79%) and motor fluctuations (73%).

Molecular Studies

Haplotype analysis of the PARK2 region was performed in 8 families, and in 3 of them, the PARK2 locus was excluded. In the 5 remaining families, haplotype analyses supported a causal role of *parkin*, and they were included in the mutational screening. Haplotype analysis could not be performed in 9 families because DNA samples from additional family members were not available.

Homozygous Deletions and Point Mutations.

Homozygous exon deletions spanning 1 to 3 consecutive exons were found in eight probands, including exons 2, 3, 5, 6, 7, 8, 9, and 10 (Table 2). One patient carried a novel deletion involving exons 8, 9, and 10.

Direct sequencing revealed several *parkin* variants, including two novel intronic changes (IVS11-3C>G and IVS2-18T>A) and two novel variants in exon 11: 1305C>T predicted to cause the amino acid change Arg402Cys, and 1353T>C leading to Cys418Arg. The known missense mutations Arg42Pro in exon 2 and Thr415Asn in exon 11 and a novel synonymous change in exon 4 620G>A (Thr173Thr) were also detected. The known polymorphisms⁵ 1239G>C (Val380Leu), 1281G>A (Asp394Asn), IVS2+25T>C, IVS3-20C>T, IVS7-35A>G were also repeatedly found.

The novel IVS11-3C>G change was found in the index case from a consanguineous Cuban family; haplotype analysis excluded the PARK6 and PARK7 loci (not shown) and suggested the possibility of compound heterozygous *parkin* mutations, because all 3 patients

TABLE 2.
Mutational screening of the parkin gene

Index case*	Presentation	Age at onset (yr)	Disease duration (yr)	<i>parkin</i> mutation 1	<i>parkin</i> mutation 2
Hom exon deletions					
TOR-34 (3, 1)	F	41, 42, 43	12, 14, 18	Exon 2–3 del	Exon 2–3 del
PK-09-01 (2, 2)	F (C)	20, 20	17, na	Exon 3 del	Exon 3 del
TOR-18 (3, 2)	F	38, 42, na	14, 28, na	Exon 5 del	Exon 5 del
IVR-1 (3, 1)	F (C)	20, 22, 29	23, na, na	Exon 5–6 del	Exon 5–6 del
PG-001 (3, 1)	F	23, 25, 25	36, 50, na	Exon 6 del	Exon 6 del
PAL-1	S (C)	18	16	Exon 6–7 del	Exon 6–7 del
ME-03 (2, 2)	F	29, 40	19, 10	Exon 8 del	Exon 8 del
PV-24	S	20	19	Exon 8–9–10 del	Exon 8–9–10 del
Het exon deletions					
Ayd01 (3, 3)	F	34, 40, 44	6, 10, 10	Exon 2 del	Exon 3–4 del
GE-01 (2, 2)	F	31, 30	33, 28	Exon 3 del	Exon 3–4 del
Het exon del / het point mutation					
RM-417	S	16	30	Exon 3 del	1345C>A (Thr415Asn)
VER-1	S	15	21	Exon 3 del	1353T>C (Cys418Arg)
Cu03 (3, 3)	F (C)	17, 23, 30	30, 16, 6	Exon 3–4 del	IVS11-3C>G (Splicing)
MI-006-01	S	28	34	Exon 6 del	226G>C (Arg42Pro)
Het point mutation					
RV-3	S	35	18	–	1305C>T (Arg402Cys)

*Number of affected, number of tested siblings in parentheses.

F, familial form; S, sporadic; C, consanguinity; del, deletion; Het, heterozygous; Hom, homozygous; na, not available.

shared haplotypes at the PARK2 locus (Fig. 1), without evidence of homozygosity.

The IVS11-3C>G change introduces a new cutting site for the restriction enzyme BseRI, and it was absent in 96 chromosomes from unrelated Cuban controls, indicating this change is not a common variant. All programs anticipated the abolition of the normal splicing acceptor site and the activation of the cryptic splice site $\overline{ACAG}/GAG$ to $\overline{AG}/AGGAG$. The second mutation (heterozygous deletion of exons 3–4) was found in this family by exon dosage analysis.

The remaining intronic change IVS2-18T>A, found in an Italian patient, was located further away from the splicing site. The computer programs predicted no affectation of splicing. The pathogenicity of this sequence change remains doubtful, and a second mutation was not found in this patient.

The novel mutations Arg402Cys and Cys418Arg were found in heterozygous state (Table 2), they are located close and within the second RING finger motif of the parkin protein and both affected highly conserved amino acids, suggesting they are pathogenic. However, in the patient carrying the Arg402Cys change, a second mutation was not found by the methods used in this study. This change was found in 1 of 500 control chromosomes (320 and 180 chromosomes of Italian and Dutch origin, respectively). The Arg42Pro mutation, located within the ubiquitin-like domain of the protein, and the Thr415Asn mutation were detected previously in homozygous state in Italian EOP families.^{5,30}

Exon Dosages Analysis.

Six index cases carried heterozygous exon rearrangements. These include 4 of the 5 probands carrying heterozygous point mutations, and 2 probands carrying two different heterozygous exon rearrangements (Table 2). In 3 families (Ver-01, Cu03, Ayd01) cosegregation and phase of the mutations could be resolved by testing other family members, delineating the patients as compound heterozygous carriers of *parkin* mutations.

In the Turkish family (Ayd01), haplotype analysis showed parental nontransmission of alleles for one *parkin* intragenic marker (D6S1599), raising the possibility of a deletional event. Real-time PCR analysis of the family delineated the 3 patients as compound heterozygous for two exon deletions involving exon 2 and exons 3–4 (Fig. 1).

Frequency of *parkin* Mutations.

We found *parkin* mutations in 15 of 46 index cases (33%, Table 2), including 53% (9 of 17) of the familial and 21% (6 of 29) of the isolated cases. Among the 15 patients with *parkin* mutations, 8 carried homozygous exon deletions, 2 were compound heterozygous for two exon deletions, and 4 carried heterozygous exon deletion plus heterozygous point mutation. In 2 of these 4 cases (RM-417, MI-006-01), the phase of the mutations remains unknown.

In one case, we found only one heterozygous missense mutation. Homozygous and heterozygous exon deletions represented 55% (16 of 29) and 28% (8 of 29) of the

FIG. 1. The pedigrees from a Turkish (Ayd01, **A**) and a Cuban family (Cu03, **B**) are shown. Filled black symbols represent the patients with early-onset Parkinson's disease. Haplotypes for the PARK2 region are displayed; alleles between brackets correspond to inferred genotypes. In pedigree A, for marker D6S1599, hemizygosity was observed; the missing allele is represented with an "X." The bar graphs below the pedigrees are showing the results from the exon dosage assay for the corresponding individuals.

observed mutant alleles, respectively. The point mutations represented 17% (5 of 29 alleles) of the *parkin* mutations; they were all heterozygous and found in patients with AAO ≤ 35 years.

Genotype-Phenotype Correlations.

The patients carrying *parkin* mutations have an earlier onset ($P = 0.02$) and longer disease duration ($P = 0.005$) than those without *parkin* mutations (Table 1). This difference originated mainly from the patients carrying a point mutation (missense or splicing), in whom we observed a mean AAO of 23 ± 8 years ($n = 7$) vs. 31 ± 9 years ($n = 16$) in the group with two exon deletions and 39 ± 10 years ($n = 37$) in the patients without *parkin* mutations (P for trend = 0.002). A similar effect was observed for the disease duration; patients with point mutations have the longest disease duration, 22 ± 10 years, vs. 19 ± 9 and 13 ± 8 years for the group with exon deletions or no *parkin* mutations, respectively (P for trend = 0.002). Although these results are statistically significant, they are based on small numbers and, therefore, should be interpreted with caution. However, the data suggest an influence of the nature of mutation on the AAO.

The clinical features in patients with and without *parkin* mutations were comparable, except for the asymmetry of signs at onset, which was less frequent in the patients with mutations ($P = 0.02$; Table 1). After adjusting for disease duration, we observed a slower disease progression in the patients with *parkin* mutations looking at the UPDRS Motor scale (41 ± 20.5 vs. 53 ± 22 , $P = 0.057$) and Hoehn and Yahr scale measured in *off* status (2.9 ± 0.9 vs. 3.4 ± 0.9 , $P = 0.006$). L-Dopa-induced motor fluctuations were more frequent in the group without *parkin* mutations who also have higher doses of L-dopa (587 vs. 497 mg), but these differences were not significant.

DISCUSSION

We have characterized clinically and genetically a series of 46 EOP index cases plus 14 affected relatives, identifying 15 different *parkin* mutations in 15 index cases, including the first Cuban family with EOP due to *parkin* mutations. Three of the five point mutations identified are novel: Arg402Cys, Cys418Arg, and IVS11-3C>G.

Recent functional studies suggest that the Cys418Arg mutation is pathogenic, because it decreases parkin solubility in cells and leads to the formation of cytoplasmic aggregates.³¹ On the contrary, whether the Arg402Cys variant is a rare polymorphism or a pathogenic mutation remains unclear and, further, functional studies might clarify this issue.

To our knowledge, only four splicing mutations^{15,16,32} have been reported in the *parkin* gene. For the IVS11-3C>G mutation reported here, five different computer programs consistently predicted the abolition of the natural acceptor splicing site and the activation of a cryptic site that competes with the authentic one, leading to a 2-bp frameshift in the sequence of exon 12. In this consanguineous Cuban family, the presence of a heterozygous exon 3–4 deletion in *trans* with the IVS11-3C>G change, illustrates the occurrence of compound heterozygous mutations in consanguineous pedigrees.

Semiquantitative and quantitative methods have been used for determination of exon dosages in the *parkin* gene. The first is based on the peak heights corresponding to each of the exons amplified in a given reaction, compared the peak heights of the control gene exon, obtained after assuming the log–linear phase of the multiplex reactions.⁶ On the other hand, quantitative methods, i.e., LightCycler, TaqMan, offer a precise (real-time) measurement of the threshold cycle. All methods used to date use expensive fluorescent primers or probes in multiplex reactions.^{6,7,10,13}

Here we describe a novel, cost-effective technique for a rapid and accurate detection of exon rearrangements in the *parkin* gene, using an intercalating dye (SYBR Green I), which functions as a fluorescent reporter, and nonlabeled primers. The amplification reaction is done independently for both sets of primers (*parkin* and β -globin) using the same master mix and same starting amount of DNA. Because this method uses only one fluorescent reporter, multiplex reaction cannot be performed. The advantage of the lower starting costs, therefore, needs to be balanced toward the throughput of a given study design, and this assay is predicted to be especially convenient for low- or moderate-throughput screenings.

Positive controls (i.e., parents and offspring of patients with homozygous deletions) were used in the respective experiments to confirm the results and validate the method. Segregation analysis in available family members allowed the identification of the allele phases and at the same time served as “quality controls.” We also confirmed all exon rearrangements compromising only one exon with an independent set of primers, to avoid false-positive results due to primer mismatch.

Heterozygous exon rearrangements represent 28% of the *parkin* mutant alleles in our study, confirming the importance of exon dosage when studying *parkin*. The detected exon rearrangements were all deletions, confirming that they are more frequent than duplications.^{6,16}

The novel method for gene copy dosage implemented here can be applied to other genes, including α -synuclein, *DJ-1*, and *PINK1*.

The frequencies of *parkin* gene mutations found in this study are consistent with previous studies that applied similar inclusion criteria but a different method for exon dosage: 49% for familial and 15% for isolated EOP patients.^{6,10} Among our isolated patients, mutations were found in 67% of the patients with a disease onset $\leq$ 20 years old, in 14% and 6% of the patients with AAO between 21 and 30 years and $\geq$ 31 years, respectively, confirming that the earlier the AAO, the higher the probability of carrying *parkin* mutations. Other studies have detected a lower frequency of *parkin* mutations (18% of all EOP patients), but they did not perform exon dosage assays.¹⁵

Previous studies suggested that a single *parkin* mutation might sometimes cause EOP or represent a risk factor for late-onset PD.^{8,12,15,16,33} Our results suggest that this issue is of minor importance in EOP, as we detected only one patient with a single heterozygous mutation (Arg402Cys), yet this may still be a rare polymorphism.

Our patients with parkin disease showed a significantly earlier age at onset, longer disease duration, more frequently symmetric onset, and slower disease progression than those without *parkin* mutations, confirming previous findings.^{6,12,33} Recently, a more severe disease status was reported in carriers of one missense mutation compared to carriers of two truncating mutations.¹² Moreover, it has been suggested that missense mutations within the functional domains of the parkin protein led to earlier AAO.¹² We observed an earlier AAO in patients with point mutations (missense and splicing) compared to patients with exon deletions or those without *parkin* mutations. These potential relationships deserve further investigation in larger sample sets.

Acknowledgments: We acknowledge the financial support from the Prinses Beatrix Fonds (The Netherlands), the Ministero dell’Istruzione, Università e Ricerca (MIUR, Italy), the IRCCS “Mondino” (Italy), and the Parkinson Disease Foundation/National Parkinson Foundation (PDF/NPF, USA). The DNA samples contributed by the Parkinson Institute—Istituti Clinici di Perfezionamento, Milan, Italy, were from the “Human genetic bank of patients affected by Parkinson disease and parkinsonisms,” supported by Telethon (GTF03009). We thank B. de Graaf for technical support, and Dr. M. Periquet and Prof. A. Brice for providing DNA samples with *parkin* exon duplications used as positive controls. Dr. Gallai is deceased.

APPENDIX

The members of the Italian Parkinson Genetics Network are as follows: Vincenzo Bonifati, Nicola Vanacore, Editto Fabrizio, Nicoletta Locuratolo, Luigi Martini, Laura Vacca, Francesca De Pandis, Carlo Colosimo, Fabrizio Stocchi, Giovanni Fabbrini, Mario Manfredi, Giuseppe Meco, University La Sapienza, Roma; Leonardo Lopiano, Alessia Tavella, Bruno Bergamasco, University of Torino; Emilia Martignoni, Cristina Tassorelli, Claudio Pacchetti, Giuseppe Nappi, IRCCS Mondino, Pavia; Stefano Goldwurm, Angelo Antonini, Gianni Pezzoli, Parkinson Institute, Istituti Clinici di Perfezionamento, Milan; Daniela Calandrella, Giulio Riboldazzi, Insubria University, Varese; Giulia de Rosa, Giancarlo Ferrari, Hospital of Ivrea; Roberto Tarletti, Roberto Cantello, University A. Avogadro, Novara; Emiliana Fincati, University of Verona; Alessio Dalla Libera, Boldrini Hospital, Thiene; Giovanni Abbruzzese, Roberta Marchese, University of Genova; Pasquale Montagna, Cesa Scaglione, Paolo Martinelli, University of Bologna; Paolo Marini, Francesca Massaro, University of Firenze; Roberto Marconi, Misericordia Hospital, Grosseto; Marco Guidi, INRCA Institute, Ancona; Chiara Minardi, Fabrizio Rasi, Bufalini Hospital, Cesena; Virgilio Gallai, Alessia Lanari, University of Perugia; Pierluigi Brustenghi, Foligno Hospital, Foligno; Ferdinando Squitieri, Milena Cannella, IRCCS Neuromed, Pozzilli; Michele De Mari, Cosimo Di Roma, Gianni Iliceto, Paolo Lamberti, University of Bari; Vincenzo Toni, Giorgio Trianni, Giulio Coppola, Hospital of Carasano; Alfonso Mauro, Hospital of Salerno; Antonio De Gaetano, Hospital of Castrovillari; Antonio De Leo, "Piemonte" Hospital, Messina.

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coordinators, located at 59 different academic sites, who completed a uniform clinical assessment of the 767 patients with Parkinson's disease.

Conflict of interest statement

We declare that we have no conflict of interest.

Acknowledgments

This project was supported by NS37167, AG18736, and M01 RR-00750. CP-R is a recipient of an FPI fellowship from the Ministerio de Educación y Ciencia (GEN2001-4851-C06-01). We thank Dr Ira Shoulson for his leadership in this collaborative study and the participants for their involvement.

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

Lancet 2005; **365**: 412–15

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Published online

January 18, 2005

<http://image.thelancet.com/extras/04let12084web.pdf>

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Mutations in the *LRRK2* gene have been identified in families with autosomal dominant parkinsonism. We amplified and sequenced the coding region of *LRRK2* from genomic DNA by PCR, and identified a heterozygous mutation (Gly2019Ser) present in four of 61 (6.6%) unrelated families with Parkinson's disease and autosomal dominant inheritance. The families originated from Italy, Portugal, and Brazil, indicating the presence of the mutation in different populations. The associated phenotype was broad, including early and late disease onset. These findings confirm the association of *LRRK2* with neurodegeneration, and identify a common mutation associated with dominantly inherited Parkinson's disease.

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

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

absent, and unusual inclusions or pathological findings usually associated with different neurodegenerative diseases are present.⁴

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

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

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

Figure: Clinical and molecular findings

(A) Simplified pedigrees of families with *LRRK2* mutations. Black symbols denote individuals affected by Parkinson's disease. Age at onset of disease or at examination shown in years. To protect confidentiality, sex of individuals is disguised and mutation carriers among youngest relatives are not indicated. One spouse (NE104) was also affected by Parkinson's disease (sporadic form), and did not carry the Gly2019Ser mutation. M=carrier of heterozygous Gly2019Ser mutation. WT=wild type genotype. NA=DNA not available. (B) Alignment of human dardarin protein and closest homologues: *Rattus norvegicus* (GenBank accession number XP_235581), *Mus musculus* (AAH34074), *Gallus gallus* (XP_425418), and *Tetraodon nigroviridis* (CAG05593). The mutated residue G2019 is highlighted. (C) Alignment of catalytic domains of human protein kinases. Asterisks indicate part of the activation segment. 1JNK=C-JUN N-terminal kinase. 1F3M=human serine-threonine kinase PAK1. 1TK1=serine kinase domain of the giant muscle protein titin. Consensus: consensus sequence for human protein kinase catalytic domain.

done in 25 μ L containing 1 $\times$ Invitrogen PCR buffer, 1.5 mmol/L $MgCl_2$, 0.01% W1 detergent, 25 μ mol/L of each dNTP, 0.4 μ mol/L forward primer, 0.4 μ mol/L reverse primer, 2.5 units of Taq DNA polymerase (Invitrogen Corporation, Carlsbad, CA, USA), and 50 ng genomic DNA. Cycle conditions were: 5 min at 94°C; 30 cycles of 30 s denaturation at 94°C; 30 s annealing; and 90 s extension at 72°C; final extension 5 min at 72°C (primers and annealing temperatures reported in the webtable, <http://image.thelancet.com/extras/04let12084>

webtable.pdf). Direct sequencing of both strands was done with Big Dye Terminator chemistry version 3.1 (Applied Biosystems, Foster City, CA, USA). Fragments were loaded on an ABI3100 automated sequencer and analysed with DNA Sequencing Analysis (version 3.7) and SeqScape (version 2.1) software (Applied Biosystems).

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

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

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

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

Novel variants that co-segregated with disease were tested in a panel of 250 chromosomes from healthy Italian people aged older than 60 years, by use of allelic specific oligohybridisation. For the Gly2019Ser mutation, PCR products containing *LRRK2* exon 41 were blotted into Hybond-N+ membranes (Amersham Biosciences, Buckinghamshire, UK). The blots were hybridised for 1 h at 37°C in 5× sodium chloride/sodium phosphate/EDTA (SSPE), 1% sodium dodecyl sulphate, and 0.05 g/L single-strand salmon sperm DNA with either the normal or mutated sequence oligonucleotides (wild-type allele: tgactacggcattg; mutant allele: gactacagcattgc). Filters were washed in buffer containing 0.045 mol/L sodium chloride, 0.0045 mol/L sodium citrate, and 0.1% sodium dodecyl sulphate, at 37°C.

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

Direct sequencing of exon 41 in the remaining 46 probands identified another two heterozygous carriers, bringing the prevalence of the Gly2019Ser mutation to four of 61 autosomal dominant families (6.6%, 95% CI 0.4–12.8).

16 individuals in these four families had Parkinson's disease, but accurate clinical information was available for only ten of them (table). These individuals had a

broad range of age of disease onset (table; average 50.5 years, range 38–68, n=10), including two patients with onset before age 40 years. All patients responded well to levodopa. Dementia and additional neurological signs were not present. Asymmetric onset and complications typically associated with long-term treatment with levodopa (motor fluctuations and choreic dyskinesias) were noted in some patients, lending support to the accuracy of the clinical diagnosis of typical Parkinson's disease.⁵ The broad range of ages of onset suggests that factors other than the mutation identified have a role in modifying the disease. Clinical features in patients who carried the Gly2019Ser mutation were similar to those of patients who did not (data not shown).

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

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

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

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Conflict of interest statement

We declare that we have no conflict of interest.

Acknowledgments

We thank the patients and family relatives for their contribution. This study was funded by grants from the National Parkinson's Disease Foundation (USA) and the Internationaal Parkinson's Fonds (Netherlands) to V Bonifati. The sponsors of the study had no role in study design, data collection, data analysis, data interpretation, or writing of the report. The corresponding author had full access to all the data in

the study and had final responsibility for the decision to submit for publication.

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A common *LRRK2* mutation in idiopathic Parkinson's disease

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Lancet 2005; **365**: 415–16

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Published online
January 18, 2005
<http://image.thelancet.com/extras/04let12032web.pdf>

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Mutations in the leucine-rich repeat kinase 2 (*LRRK2*) gene have been shown to cause autosomal dominant Parkinson's disease. Few mutations in this gene have been identified. We investigated the frequency of a common heterozygous mutation, 2877510G→A, which produces a glycine to serine aminoacid substitution at codon 2019 (Gly2019Ser), in idiopathic Parkinson's disease. We assessed 482 patients with the disorder, of whom 263 had pathologically confirmed disease, by direct sequencing for mutations in exon 41 of *LRRK2*. The mutation was present in eight (1.6%) patients. We have shown that a common single Mendelian mutation is implicated in sporadic Parkinson's disease. We suggest that testing for this mutation will be important in the management and genetic counselling of patients with Parkinson's disease.

Although Parkinson's disease is a common neurodegenerative condition, the disease trait is rarely inherited in a simple Mendelian fashion. However, the study of families with inherited Parkinson's disease has greatly improved our knowledge of the genetic and molecular basis of this incurable disorder.¹ We have shown that a form of autosomal dominant Parkinson's disease (PARK8) was caused by mutations in the *LRRK2* gene (MIM 609007) in a British family and several Basque families.² We have subsequently identified a common missense mutation in four patients with familial Parkinson's disease (unpublished data). These individuals harboured a heterozygous 2877510G→A change that causes a Gly2019Ser substitution (GeneBank AAV63975) adjacent to a previously reported Iso2020Thr mutation in a highly conserved region of the predicted kinase domain.³

This finding prompted us to investigate the frequency of the Gly2019Ser mutation in idiopathic Parkinson's disease. We screened 482 patients with sporadic

Parkinson's disease (263 had pathologically confirmed disease) by direct sequencing for mutations in exon 41 of *LRRK2*. We did not screen this series for any other mutations in *LRRK2*. All patients and controls were of white ancestry, predominantly from the south-east of England, and were recruited through the National Hospital for Neurology and Neurosurgery. Patients with Parkinson's disease were diagnosed clinically or pathologically from the Queen Square Brain Bank for Neurological Disease, satisfying rigorous accepted diagnostic criteria.⁴ Of the 345 controls, 102 samples were from unaffected and unrelated relatives of patients with Huntington's disease, and had been obtained for linkage analysis studies. The remaining 243 samples were from unrelated patients who had been genetically diagnosed with non-parkinsonian disorders (eg, mitochondrial myopathies, inherited neuropathies). The study was approved by the Joint Research Ethics Committee of the Institute of Neurology and The National Hospital for Neurology and Neurosurgery, London, UK. Informed written consent was obtained from all patients.

Genomic DNA was extracted from peripheral blood leucocytes or brain cortex tissue by a semi-automated method (Kurabo, Osaka, Japan). PCR products were generated with 50 ng DNA template in 2.5 µL buffer,

Panel: Primers

Forward: 5'TTTTGATGCTTGACATAGTGGAC3'
Reverse: 5'CACATCTGAGGTCACTGGTTATC3'

Early-onset parkinsonism associated with *PINK1* mutations

Frequency, genotypes, and phenotypes

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Abstract—Objective: To assess the prevalence, nature, and associated phenotypes of *PINK1* gene mutations in a large series of patients with early-onset (<50 years) parkinsonism. **Methods:** The authors studied 134 patients (116 sporadic and 18 familial; 77% Italian) and 90 Italian controls. The whole *PINK1* coding region was sequenced from genomic DNA; cDNA was analyzed in selected cases. **Results:** Homozygous pathogenic mutations were identified in 4 of 90 Italian sporadic cases, including the novel Gln456Stop mutation; single heterozygous truncating or missense mutations were found in another 4 Italian sporadic cases, including two novel mutations, Pro196Leu and Gln456Stop. Pathogenic mutations were not identified in the familial cases. Novel (Gln115Leu) and known polymorphisms were identified with similar frequency in cases and controls. In cases carrying single heterozygous mutation, cDNA analysis detected no additional mutations, and revealed a major pathogenic effect at mRNA level for the mutant C1366T/Gln456Stop allele. All patients with homozygous mutations had very early disease onset, slow progression, and excellent response to L-dopa, including, in some, symmetric onset, dystonia at onset, and sleep benefit, resembling parkin-related disease. Phenotype in patients with single heterozygous mutation was similar, but onset was later. **Conclusions:** *PINK1* homozygous mutations are a relevant cause of disease among Italian sporadic patients with early-onset parkinsonism. The role of mutations found in single heterozygous state is difficult to interpret. Our study suggests that, at least in some patients, these mutations are disease causing, in combination with additional, still unknown factors.

NEUROLOGY 2005;65:87–95

The importance of genetic susceptibility is increasingly recognized among patients with Parkinson disease (PD) with onset before the age of 50 years (early-onset PD), and three loci for autosomal recessive parkinsonism are known, termed PARK2, PARK6, and PARK7.^{1,2}

Additional material related to this article can be found on the *Neurology* Web site. Go to www.neurology.org and scroll down the Table of Contents for the July 12 issue to find the title link for this article.

The PARK6 locus was mapped in a Sicilian family,³ and later confirmed in European and Asian families.^{4,5} The associated pathology remains unexplored. Recently, homozygous pathogenic mutations in the *PINK1* gene (PTEN-induced putative kinase 1) were identified in PARK6-linked families.⁶

The *PINK1* gene encodes a 581 amino acid protein of unknown function, with an N-terminal mitochondrial targeting peptide and a putative Ser/Thr kinase

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Supported by the National Parkinson Foundation (USA); the Stichting Klinische Genetica Rotterdam (The Netherlands); the Ministero dell’Istruzione, Università e Ricerca (MIUR, Italy); and the IRCCS “Mondino” (Italy). The DNA samples contributed by the Parkinson Institute-Istituti Clinici di Perfezionamento, Milan, Italy, were from the “Human genetic bank of patients affected by PD and parkinsonisms,” supported by Telethon grant n. GTF03009.

Received December 12, 2004. Accepted in final form March 29, 2005.

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domain.⁶ Mitochondrial abnormalities and oxidative stress have been implicated in the pathogenesis of classic PD^{7,8}; understanding the function of PINK1 and the mechanisms of disease caused by *PINK1* mutations (PINK1-related disease) might therefore provide clues into the pathogenesis of the common forms of PD.

PINK1 mutations have been reported in other early-onset PD patients,^{9,10} but the prevalence, mutational spectrum, and phenotype of PINK1-related disease remain uncertain, as only three studies have been reported on large samples.¹¹⁻¹³ Moreover, the role of the single heterozygous *PINK1* mutations in early-onset PD remains unclear.

We sequenced the *PINK1* coding region from genomic DNA in 134 consecutive early-onset PD cases and 90 controls. In the patients with a single heterozygous mutation detected by this method, we also performed cDNA analysis.

Methods. A total of 134 patients, representing two cohorts of consecutively collected familial and sporadic cases, were studied.

Eighteen probands were from PD families compatible with autosomal recessive inheritance (≥ 2 affected siblings and unaffected parents) and early onset (average: 36 years of age, range 10 to 50) (AR cohort). Among these families, 13 were from Italy, 3 from Brazil, and 2 from The Netherlands. This cohort was selected from a larger sample of 34 early-onset AR families, after the *parkin* (*PARK2*) and *DJ-1* (*PARK7*) genes were screened (by gene sequencing and dosage), and the families with mutations ($n = 16$) were removed.

A total of 116 cases with early-onset PD (S cohort) were classified as sporadic because they reported no first-degree relatives with PD. However, 9 of these 116 cases reported one second-degree relative with PD, 2 cases reported one third-degree relative with PD, and 3 cases reported one first-degree relative with tremor only. In this cohort, the average onset age was 36.1 years (range 18 to 45). Three of the sporadic cases had onset before 20 years of age, 16 between 21 and 30 years, 73 between 31 and 40 years, and 24 between 41 and 45 years. Among the sporadic cases, 90 were from Italy, 12 from the United Kingdom, 7 from The Netherlands, 5 from Brazil, and 1 each from Uruguay and Morocco. The *parkin* and *DJ-1* genes were not tested in most cases from this cohort, with the exception of the 12 UK cases, in which *parkin* mutations had been excluded (by gene sequencing and dosage).

The parents of patients were consanguineous in 3 of the 18 families and in 8 sporadic cases.

The clinical diagnosis of definite PD required the presence of bradykinesia and at least one of the following: resting tremor, rigidity, and postural instability; a positive response to dopaminergic therapy; the absence of atypical features or other causes of parkinsonism.¹⁴ The patients displaying the same clinical features but still untreated with dopaminergic drugs were diagnosed with clinically likely PD. Neurologic examination included the Unified PD Rating Scale (UPDRS, motor part) and Hoehn-Yahr scale.

All but two patients received a clinical diagnosis of PD (125 definite PD, 7 likely PD): 20 familial cases (1 Italian and 1 Dutch) with juvenile onset (< 20 years) displayed a broader clinical phenotype, involving the extrapyramidal and pyramidal systems and resembling the pallido-pyramidal degeneration.

The *PINK1* coding changes detected in the patients (located in exon 2 and exon 7) were tested by direct sequencing in 130 healthy, unrelated Italian subjects aged > 60 years (260 alleles). These subjects had neither PD nor first-degree relatives with PD. In 90 of these subjects the complete coding region of *PINK1* was also sequenced (exon 1 to exon 8).

PINK1 genomic and cDNA analysis. Written informed consent was obtained from all subjects. Genomic DNA was isolated from peripheral blood using standard protocols. The eight exons of the *PINK1* gene were amplified using PCR and intronic primers. For exon 1, two overlapping fragments were amplified (ex.1A and

ex.1B). Primer sequences and PCR protocols are reported in table E-1 (on the *Neurology* Web site at www.neurology.org). Direct sequencing of both strands was performed using Big Dye Terminator chemistry version 3.1 (Applied Biosystems). Fragments were loaded on an ABI3100 automated sequencer and analyzed with DNA Sequencing Analysis (version 3.7) and SeqScape (version 2.1) software (Applied Biosystems). The consequences of the mutation at the protein level were predicted according to the *PINK1* mRNA sequence (accession number NM_032409).

Total mRNA was isolated from EBV-transformed lymphoblastoid cell lines, or from peripheral blood, and cDNA was prepared using Superscript-II and reverse transcriptase PCR (RT-PCR), according to standard protocols. Samples from the following subjects were available for cDNA analyses: five patients carrying single heterozygous *PINK1* mutations (C587T/Pro196Leu, C1366T/Gln456Stop [two cases], G1426A/Glu476Lys, and IVS5-4 C/T) and some unaffected relatives; the patient carrying the homozygous mutation 1573_1574insTTAG (Asp525frameshiftStop562) we reported recently,¹⁰ and unaffected relatives; one patient carrying a novel coding polymorphism identified in this study (A344T/Gln115Leu) and unaffected relatives.

A cDNA fragment of 1744 base pairs, spanning exon 1 to exon 8 of the *PINK1* transcript, was amplified from the RT-PCR material using TaKaRa LA Taq polymerase and GC PCR buffer (Takara Biomedicals). Primer sequences and PCR conditions used are reported in table E-1. cDNA fragments were directly sequenced as described above for genomic DNA.

Frequencies of polymorphisms in cases and controls were compared using the χ^2 test with Fisher's correction, where needed.

Bioinformatics of the PINK1 protein. The closest homologues of the PINK1 protein were identified by blasting the human sequence using the BLASTp program. Retrieved sequences were saved in FASTA format and aligned using the program ClustalW and the European Bioinformatic Institute server (<http://www.ebi.ac.uk/clustalw/index.html>). GenBank accession numbers for the PINK1 protein homologues are as follows: NP_115785.1 (*Homo sapiens*); BAB64474.1 (*Macaca fascicularis*); XP_216565.2 (*Rattus norvegicus*); NP_081156.1 (*Mus musculus* #1, strain "ICR"); AAH67066.1 (*Mus musculus* #2, strain C57BL/6); BAB55651.1 (*Mus musculus* #3, strain C57BL/6Njcl); XP_423139.1 (*Gallus gallus*); XP_313587.1 (*Anopheles gambiae*); NP_727110.1 (*Drosophila melanogaster*); NP_495017.1 (*Caenorhabditis elegans*).

Results. Genomic DNA studies. Genomic sequencing of *PINK1* in the patients revealed the changes detailed in table 1. Homozygous truncating or missense mutations were found in four Italian sporadic patients. One case, carrying the novel truncating mutation Gln456Stop, is reported here for the first time; another case, carrying the truncating mutation Asp525fsStop562, was reported by us recently.¹⁰ The two remaining cases carry homozygous missense mutations, Ala168Pro and Trp437Stop, which were previously reported by others in different patients.^{6,12}

Another four sporadic Italian patients carried a single heterozygous truncating or missense mutation; these include the novel missense mutation Pro196Leu (one case) and the novel truncating Gln456Stop (two cases); the remaining case carries the Glu476Lys missense mutation previously reported by others.^{12,13}

Furthermore, two sporadic Italian patients carried novel, single heterozygous intronic mutations, whose biologic effect is unknown: IVS3 + 38_+40delTTT, and IVS5-4C/T.

Mutations were not identified in the cohort of AR families, or in the smaller groups of sporadic cases originating from UK, Brazil, The Netherlands, Uruguay, and Morocco.

In two Italian familial cases (see table 1) novel, single heterozygous variants were found, which did not cosegregate with PD in the families, and were therefore considered rare disease-unrelated changes: Asp391Asp (silent variant in exon 6), and IVS6 + 22C/T.

Table 1 *PINK1* mutations found in this study

	Nucleotide change	Codon effect	Protein effect	Patient code or no. of subjects
Sporadic patients				
Homozygous				
Ex.2	G502C	GCT→CCT	Ala168Pro	NE-157
Ex.7	G1311A	TGG→TGA	Trp437Stop	CS-07
Ex.7*	C1366T*	CAG→TAG*	Gln456Stop*	NE-166*
Ex.8	1573_1574insTTAG	frameshift	Asp525fsStop562	Bol-22
Heterozygous truncating or missense				
Ex.2*	C587T*	CCA→CTA*	Pro196Leu*	BARI-1011*
Ex.7*	C1366T*	CAG→TAG*	Gln456Stop*	ROMA-360* MI-002-03*
Ex.7	G1426A	GAG→AAG	Glu476Lys	PV-43
Heterozygous intronic				
IVS3+38_+40delTTT*		NA*	NA*	TOR-39
IVS5-4C→T*		NA*	NA*	BARI-1018*
Familial patients				
Heterozygous intronic or silent, showing no co-segregation with disease				
Ex.6	T1173C	GAT→GAC	Asp391Asp	BO-53 family
IVS6+22C→T*		NA*	NA*	IT-250 family
Controls				
Heterozygous truncating or missense				
Ex.7	G1311A	TGG→TGA	Trp437Stop	1
Heterozygous UTR or silent				
5'UTR-82G→A*		NA*	NA*	1*
5'UTR-20C→T*		NA*	NA*	1*
Ex.4*	C852T*	TCC→TCT*	Ser284Ser*	1*

* Novel mutations.

NA = not applicable.

Genomic sequencing of the entire *PINK1* coding region in 90 Italian controls revealed four carriers of single heterozygous changes (see table 1): a previously reported truncating mutation (Trp437Stop), two novel single nucleotide variants located in the 5' untranslated region (5'UTR), 82 and 20 bases before the "A" of the ATG translation initiation codon, and a silent change in exon 4 (Ser284Ser).

The exons 2 and 7, where the truncating and coding mutations detected in the patients are located, were sequenced in an additional group of 40 Italian controls (for a total of 130 subjects, 260 chromosomes), without revealing any further mutations.

Different variants present in several cases and controls were considered as disease-unrelated, neutral polymorphisms (table 2). Among these, we identified a novel, frequent coding variant in exon 1, A344T (Gln115Leu).

***PINK1* protein analyses.** Three truncating and three missense mutations were identified in patients from our study. The missense mutations Ala168Pro and Pro196Leu replace highly conserved residues (figure 1). On the contrary, the missense mutation Glu476Lys, despite replacing a negatively charged with a positively charged residue, is targeting an amino acid which has not been highly con-

served, and Lys is replacing Glu at this position in the rat and in one out of three mouse strains examined, showing that the Glu476Lys change is a polymorphism in mice.

cDNA studies. RT-PCR experiments showed that *PINK1* mRNA is expressed in peripheral leukocytes and in lymphoblastoid cell lines (figure 2, A through C). A single cDNA fragment of the expected size (1,744 base pairs), and spanning the eight exons of *PINK1*, was amplified from RT-PCR material from the patient with the Gln115Leu polymorphism and all (five) patients with single heterozygous mutations analyzed (figure 2B). Sequencing this cDNA fragment confirmed in three of these patients the heterozygosity identified by genomic sequencing at the position of the point mutation and at other polymorphic sites (see figure 2, A and C). Taken as a whole, the results of the cDNA studies indicate that two *PINK1* alleles are expressed in peripheral blood of these patients, and strongly suggest that a second heterozygous mutation, such as genomic rearrangement (exon deletion or multiplication), or a mutation in the promoter, introns, and other regulatory elements, is absent.

In two patients (ROMA-360 and MI-002-03) cDNA sequencing revealed a homozygous C1366 nucleotide (wild type), whereas this position was heterozygous (C1366T) by

Table 2 Disease-unrelated *PINK1* frequent variants detected in Italian subjects

Seq. variation	Exon	aa Change	MAF	Cases, n = 103 (S+AR)						Controls, n = 90					
				Genotypes			Alleles		MAF	Genotypes			Alleles		
				WT/WT	WT/Var	Var/Var	WT	Var		WT/WT	WT/Var	Var/Var	WT	Var	
C189T	1	Leu63Leu	0.218	61	39	3	161	45	0.267	47	38	5	132	48	
A344T*	1	Gln115Leu	0.044	94	9	0	197	9	0.078	77	12	1	166	14	
IVS1-65C→G			0.107	82	20	1	184	22	0.156	64	24	2	152	28	
IVS1-66_63 ins/delCT			0.097	83	20	0	186	20	0.111	71	18	1	160	20	
IVS1-7G→A			0.112	81	21	1	183	23	0.156	64	24	2	152	28	
IVS4-5A→G			0.102	83	19	1	185	21	0.156	64	24	2	152	28	
G1018A	5	Ala340Thr	0.024	99	3	1	201	5	0.039	84	5	1	173	7	
IVS6+43C→T			0.010	101	2	0	204	2	0.011	88	2	0	178	2	
A1562C	8	Asn521Thr	0.209	64	35	4	163	43	0.272	45	41	4	131	49	
3'UTR+37T→A			0.112	83	17	3	183	23	0.178	63	22	5	148	32	

* Novel exon1 polymorphism detected in this study.

MAF = minor allele frequency.

genomic sequencing (see figure 2, A and C). The same cDNA pattern was confirmed in the mother of MI-002-03, also found to be a heterozygous carrier of the C1366T/Gln456Stop mutation by genomic DNA sequencing (not shown). Furthermore, in the patient MI-002-03, cDNA sequencing revealed a homozygous T189 nucleotide in exon 1, whereas this position was also heterozygous by genomic sequencing (C189T) (see figure 2A). These findings consistently suggest that the mutant T1366 allele is not expressed, or mRNA is unstable due to this mutation or to another change in linkage disequilibrium with the T1366 change. This is an example of a mutant allele exerting its major biologic effect at mRNA and not at the protein level. A second mutation could not be found in these cases either.

Due to the lack of mRNA samples, cDNA studies could not be performed in the remaining patient with single heterozygous intronic mutation (TOR-39).

Finally, cDNA analysis in the Bol-22 family (including the homozygous index case and her heterozygous parents) confirmed the zygosity detected by genomic sequencing for the 1573_1574insTTAG mutation, indicating that this mutation has no major effects on mRNA expression or stability (not shown).

Clinical studies. The most important clinical features in the patients carrying homozygous and heterozygous mutations are reported in table 3. Onset of symptoms was before age 36 years in all four cases carrying homozygous *PINK1* mutations, whereas it was after age 40 years in two

of the cases carrying single heterozygous mutations. Disease course was very slow, as shown by the small UPDRS motor scores in two of the cases with homozygous mutations, after 44 and 23 years from disease onset. Symptoms onset was asymmetric only in two of the four homozygous cases, and in all the heterozygous cases. Dystonic features at onset and sleep benefit were present in some patients with homozygous mutations. L-dopa response was good in all treated cases and L-dopa-induced motor fluctuations and dyskinesias developed in most of them. Several cases had severe anxiety requiring medication. In one case (NE-157) peripheral sensorimotor neuropathy was present in addition to parkinsonism, and another case (MI-002-03) had severe muscular fatigue. Detailed clinical reports of patients carrying homozygous and heterozygous mutations are contained in the online Appendix (available on the *Neurology* Web site at www.neurology.org).

Discussion. The prevalence of *PINK1* mutations among early-onset PD has been investigated in three recent studies.¹¹⁻¹³ One study, performed on 90 Italian sporadic early-onset cases, found mutations in a relatively high percentage of patients (7.7% total, including 2.2% with homozygous or compound heterozygous, and 5.5% with single heterozygous missense mutations).¹² The prevalence was much lower according to another study, which detected no *PINK1* homozygous or compound heterozygous mutations among 290 Irish patients (86 of whom had onset before 45 years of age). Only one single heterozygous missense mutation was found in a patient with onset at age 51.¹¹ In both studies, exon copy dosage was performed in cases with single heterozygous mutations. In the third study, 289 North American patients, of whom 165 had early onset, were included.¹³ Homozygous or compound heterozygous mutations were found in two familial early-onset

	Ala168	Pro196	Glu476
Human	162 IGKGCNNAVYEA	191 LPGRGEGTSAP	471 LPALPESVPPD
Macaque	122 IGKGCNNAVYEA	151 LPGRGEGTSAP	431 LPALPESVPPD
Rat	162 IGKGCNNAVYEA	190 LLGKGEDVVS	470 LPEMPKSVVPE
Mouse #1	162 IGKGCNNAVYEA	190 LIGKGEDVVLK	470 LPEMPKSVVPE
Mouse #2	162 IGKGCNNAVYEA	190 LIGKGEDVVLK	470 LPEMPKSVVPE
Mouse #3	162 IGKGCNNAVYEA	190 LIGKGEDVVLK	470 LPEMPKSVVPE
Drosophila	200 IARGCANVYAA	229 QPQATAFAPN	598 LPPMSDACPPL
Anopheles	168 IARGCANVYAA	197 IHNSK-SSSNG	553 LPAMDQNVRL
C. elegans	143 LGQGCNNAVYSA	172 GFNEVTNILAE	442 LPALPESRVNPF

Figure 1. Alignment of *PINK1* protein homologues at position of missense mutations found in this study.

Figure 2. *PINK1* cDNA analysis. (A) Schematic representation of *PINK1* genomic (exons are boxed) and cDNA structures. All the heterozygous exonic sites are indicated (mutations by arrows, polymorphisms by arrowheads). The long horizontal dashed arrow indicates the 1,744-bp cDNA fragment amplified by RT-PCR and used for sequencing. (B) Agarose gel showing the amplification of the 1,744-bp cDNA fragment. Lanes 1, 7: 1Kb Plus DNA ladder; lanes 2, 3, 4, 5: representative patients; lane 6: blank. (C) Electropherograms of genomic and cDNA sequences (see text for details). In two patients (ROMA-360 and MI-002-03) the heterozygosity detected by genomic sequencing could not be observed at cDNA level (red arrows and arrowheads in panels A and C).

cases and in none of the sporadic cases. Single heterozygous missense mutations were found in six cases, whose onset age and familial/sporadic pattern were not reported. Gene copy dosage was not performed either.

Our prevalence figures are the highest reported to date, and suggest that *PINK1* mutations are responsible for a small but relevant percentage of sporadic cases with early-onset parkinsonism in Italy. In our series, among 90 sporadic Italian cases with early-onset PD, four patients are conclusively explained by homozygous mutations in this gene; these are patho-

genic because they are predicted to truncate the *PINK1* protein, or to replace highly conserved residues, and are absent (at least in homozygous state) in controls. Another four Italian sporadic patients carry a single heterozygous “major” change (truncating or missense) in the coding region. Among the 90 controls, only one such variant was observed.

The fact that we found no mutations in 18 familial, early-onset AR cases in which *parkin* and *DJ-1* mutations had been excluded suggests that the prevalence of *PINK1* mutations is much lower than that of *parkin* mutations. Mutations in the *parkin* and

Table 3 Clinical features in patients with *PINK1* mutations

Patient	Homozygous coding mutants				Heterozygous coding mutants				Het. intronic mutants	
	NE-157	CS-07	NE-166	BOL-22	BARI-1011	MI-002-03	ROMA-360	PV-43	BARI-1018	TOR-39
Mutation	A168P	W437X	Q456X	D525fsX562	P196L	Q456X	Q456X	E476K	IVS5-4C/T	IVS3+38_40delTTT
Sex	M	F	F	F	M	F	M	F	F	F
Onset age, y	30	30	35	28	45	34	41	36	40	39
Duration, y	44	23	2	7	15	9	11	6	2	24
UPDRS (on/off)	26/46	19/NA	15/NA	9/NA	27/NA	10/NA	12/NA	12/NA	9/NA	37/95
Tremor	-	+	+	+	+	-	-	+	-	+
Bradykinesia	+	+	+	+	+	+	+	+	+	+
Rigidity	+	+	+	+	+	+	+	+	+	+
Postural instability	+	+	-	+	+	-	+	-	-	+
Asymmetric onset	-	+	-	+	+	+	+	+	+	+
Dystonia at onset	-	(+)	+	+	-	+	-	-	-	-
L-Dopa response	+	+	NA*	+	+	+	+	+	+	+
Motor fluctuations	+	+	NA*	+	+	-	+	+	-	+
Dyskinesias	+	+	NA*	+	+	-	+	+	-	+
Brisk reflexes	-	-	+	-	-	-	-	-	-	-
Sleep benefit	-	+	-	+	-	+	+	-	+	-
Severe anxiety	+	-	+	+	+	-	+	-	-	-
Depression	+	-	+	-	+	-	-	-	-	-
Psychosis	-	-	-	-	-	-	-	+	-	+
Dementia	-	-	-	-	-	-	-	-	-	-
Dysautonomia	-	-	-	-	-	-	-	-	-	-
Others	Peripheral neuropathy	-	-	-	-	Fatigue	-	-	-	DBS

* Untreated with L-dopa.

UPDRS = Unified Parkinson's Disease Rating Scale (motor score) in "on" and "off" condition; NA = not applicable or not available; + = present; - = absent; dyskinesias = L-dopa-induced dyskinesias; DBS = treated with deep brain stimulation.

DJ-1 gene were found in 15 and 1 of our total sample of 34 AR families. In a recent study⁹ homozygous or compound heterozygous *PINK1* mutations were found in 6 out of 39 (~15%) early-onset AR families, mostly Asian, in which *parkin* and *DJ-1* mutations had been excluded. It is possible that *PINK1*-related disease is more frequent in the Asian population than in whites. The findings from this and other studies^{11,12} also suggest that the frequency of *PINK1* mutations is higher in Italian early-onset PD patients than in patients from Northern Europe. This might have implications for the diagnostic workup and counseling of early-onset PD.

For none of the polymorphic *PINK1* variants identified did allelic and genotype frequencies differ between Italian cases and controls. In agreement with the results of other studies,^{15,16} this suggests that *PINK1* common variants are not major risk factors for early-onset parkinsonism.

We did not perform *PINK1* exon copy dosage, and might have missed large heterozygous exonic deletions or multiplications. However, homozygous dele-

tions would have been detected in this and previous studies. Taken together these results suggest that, contrary to the scenario seen for *parkin* and *DJ-1* gene, genomic *PINK1* rearrangements are rare.

The *PINK1* protein with the mutations found in PD patients in this and previous studies^{6,9,11-13} are depicted in figure 3. Fifteen homozygous or compound heterozygous *PINK1* mutations are known so far, which definitely cause early-onset parkinsonism; nine are missense and six truncating. Most mutations are within the protein kinase domain, suggesting that the loss of the *PINK1* kinase function causes this form of early-onset PD.

In five cases carrying single heterozygous mutations, we consistently found no evidence of a second mutation at the cDNA level, suggesting that these patients are truly carrying a single heterozygous mutation, at least in peripheral blood cells. In previous studies, exon copy dosage analysis on genomic DNA found no evidence of heterozygous genomic rearrangements in cases carrying single heterozygous point mutations.^{11,12}

Figure 3. *PINK1* mutations reported in patients with parkinsonism. Missense and truncating mutations are depicted above and below the protein bar. Mutations found in homozygous or compound heterozygous state are in black. Mutations found in heterozygous state are in gray. Data from this and previous studies (refs. 6, 9, 11, 12, 13).

The role of single heterozygous *PINK1* mutations in early-onset PD is difficult to interpret. More functional studies are needed in order to clarify their biologic effect. Some missense variants, like Pro196Leu, are predicted to severely disrupt the protein folding and to replace highly conserved residues, and are very likely harmful. In other cases, like for Glu476Lys or Arg147His,¹¹ the replaced residue is not highly conserved, and it cannot be excluded that these are rare benign variants. The biologic effect is also unclear for the rare intronic variants identified. In the case carrying the IVS5-4 C/T change, our cDNA analysis failed to detect splicing aberrations and suggests that this is also a neutral variant.

However, two arguments suggest that the heterozygous mutations detected in early-onset patients are not a chance finding but at least in some cases, they are causally associated with the disease. If the results of this and the previous study performed on Italian early-onset cases are combined (table 4), the prevalence of single heterozygous truncating or missense mutations is 5% among 180 cases vs 1% among 290 controls ($p < 0.01$). Moreover, the observed frequency of single heterozygous variants among cases (0.05) is higher than the frequency computed on the basis of the population prevalence of PD,¹⁷ under the assumption that 10% of PD cases have early onset,¹⁸ and that 10% of the early-onset PD are explained by *PINK1* mutations (homozygous or compound het-

erozygous). The population frequency of heterozygous carriers of *PINK1* mutation, computed in this way, falls between 0.0065 and 0.009, or 1 in about 100 to 200 individuals, and is even lower if *PINK1* is assumed to explain 5% of the early-onset cases.

The *PINK1* single heterozygous variants could act as loss-of-function mutations by lowering the biologic activity of the encoded protein to ~50% (haploinsufficiency), or they could affect the product of the other allele (dominant-negative), or act as gain-of-function dominant mutations. In all these cases, one should observe a dominant pattern of disease transmission in pedigrees, which is not the case in families with *PINK1* mutations reported to date.

We examined seven first-degree relatives of patients with homozygous mutations who are heterozygous carriers and have passed the age of disease onset in their affected relatives (see the clinical reports in supplementary Appendix E-1). We did not find early-onset parkinsonism in any of them, and in only one individual we found mild, late-onset parkinsonian signs (CS-07 father).

Previous PET studies showed evidence of decreased dopaminergic function in asymptomatic heterozygous carriers of *PINK1*- (and *parkin*-) mutations,^{19,20} suggesting that some mutations in heterozygous state harm the dopaminergic system, at least subclinically, and this might predispose to late-onset disease.

Table 4 Frequency of *PINK1* mutations in Italian sporadic early-onset cases and controls

	<i>n</i>	Homozygous or comp/heterozygous	Heterozygous missense or truncating	Heterozygous uncertain significance
This study				
Sporadic Cases (onset <45)	90	4	4	2 IVS
Controls	90	—	1	3, 2 5'-UTR, 1 silent
Data from previous study (ref.12)				
Sporadic Cases (onset <50)	90	2	5	3 silent
Controls	200	—	2	—
Both studies combined				
Sporadic Cases	180	6 (3.3 %)	9 (5.0%)*	5 (2.8%)
Controls	290	—	3 (1.0%)	3 (1.0%)

* $p < 0.01$ vs controls.

Figure 4. Distribution of onset age in patients with early-onset parkinsonism and *PINK1* mutations. Data are from this and previous studies. Onset age is not available for three cases with homozygous mutations belonging to the Spanish family included in ref. 6. For the five heterozygous cases in ref. 12, data represent mean and extreme values of the range. S = sporadic; F = familial (autosomal recessive) cases.

The fact that mutations (such as Gln456Stop) found in some early-onset patients in single heterozygous state are present in homozygous state in other cases with early-onset PD also argues against the idea that a single heterozygous *PINK1* mutation is sufficient to cause early-onset PD.

A more likely explanation is that, at least in some cases with single heterozygous *PINK1* mutation, other, still unknown factors act in combination to cause an early-onset phenotype. These factors might be mutations located elsewhere in the genome, particularly in genes encoding proteins, which act in the same pathway of *PINK1*, or might be environmental factors. The possibility that a second mutation only occurs in the brain tissue (somatic mosaicism) also remains to be explored. In our cases carrying single heterozygous *PINK1* coding mutations, the screening of the *parkin* and *DJ-1* gene revealed no mutations.

Whether single heterozygous mutations in *PINK1* are associated with late-onset PD is a different question, which requires screening of large cohorts of late-onset PD cases and controls.

The clinical phenotype in families with homozygous *PINK1* mutations was initially characterized by early onset, absence of distinguishing features at onset (such as dystonia, sleep benefit, or psychiatric disturbances), excellent and sustained response to L-dopa, slow progression, frequent (and sometimes very early) L-dopa-induced motor fluctuations, and dyskinesias. Dementia or severe symptomatic vegetative disturbances were not present.^{12,21} These features were substantially confirmed in Asian patients

with homozygous mutations, although dystonia at onset occurred in 2 of 10 cases, sleep benefit in 5 of them, and dementia was also described in 1 Israeli case.^{5,9} Our four homozygous cases (see table 3) all have early-onset, excellent L-dopa response, and very slow course; furthermore, symmetric onset, dystonia at onset, and sleep benefit were present in few. Motor fluctuations and dyskinesias were also very common. We did not observe dementia or severe vegetative disturbances, but severe anxiety was a common feature in homozygous (and heterozygous) patients in our series. The phenotype in the group of cases with homozygous *PINK1* mutations appears therefore broad, and in some cases, very similar to the phenotype of parkin- and DJ-1-related disease.

Results from the comparison between the group of cases with homozygous and heterozygous mutations must be interpreted with caution, also because of the small numbers available so far. However, on the basis of the data available from this and previous studies,^{9,11-13,21} the clinical features are broadly similar; onset age shows a wide variation in both groups (figure 4), but it was about 10 years earlier in the homozygous patients (mean: 31 years, range 18 to 48, n = 20) than in the heterozygous ones (mean 42.7, range 34 to 51, n = 10). Larger numbers are also needed to make comparisons between cases with missense vs truncating mutations.

In one of our cases (NE-157), carrying the Ala168Pro homozygous mutation, sensory-motor neuropathy was also present, expanding further the phenotype associated with *PINK1* mutations. Neu-

ropathy has been reported in cases with parkin-related disease,²²⁻²⁵ suggesting that the degenerative process in parkin- and PINK1-related disease might sometimes include the peripheral nervous system.

Our findings delineate PINK1-related disease as a relevant cause of early-onset parkinsonism in the Italian population, and expand its mutational spectrum and the associated clinical phenotype. There are no clinical features, which allow cases with PINK1 mutations to be distinguished from those with mutations in other genes (*parkin* or *DJ-1*) or those without mutations. Genetic testing is therefore essential for an accurate diagnosis and distinction between the different recessive forms of early-onset parkinsonism.

Acknowledgment

The authors thank the patients and relatives for their contributions, and Tom de Vries-Lentsch for artwork.

Appendix

The members of the Italian Parkinson Genetics Network are as follows: V. Bonifati, N. Vanacore, E. Fabrizio, N. Locuratolo, L. Martini, C. Scoppetta, G. Fabbri, M. Manfredi, G. Meco, *University "La Sapienza," Rome*; L. Lopiano, A. Tavella, B. Bergamasco, *University of Torino*; E. Martignoni, C. Tassorelli, C. Pacchetti, G. Nappi, *IRCCS "Mondino," Pavia*; S. Goldwurm, A. Antonini, G. Pezzoli, *Parkinson Institute, Istituti Clinici di Perfezionamento, Milan*; D. Calandrella, G. Riboldazzi, G. Bono, *Insubria University, Varese*; R. Tarletti, R. Cantello, *University "A. Avogadro," Novara*; M. Manfredi, *"Poliambulanza" Hospital, Brescia*; E. Fincati, *University of Verona*; M. Tinazzi, A. Bonizzato, *Hospital "Borgo Trento," Verona*; A. Dalla Libera, *"Boldrini" Hospital, Thiene*; G. Abbruzzese, R. Marchese, *University of Genova*; P. Montagna, *University of Bologna*; P. Marini, F. Massaro, *University of Firenze*; R. Marconi, *"Misericordia" Hospital, Grosseto*; M. Guidi, *"INRCA" Institute, Ancona*; C. Minardi, F. Rasi, *"Bufalini" Hospital, Cesena*; P. Brustenghi, *Hospital of Foligno*; L. Vacca, F. Stocchi, *IRCCS Neuro-med, Pozzilli*; F. De Pandis, *"Villa Margherita" Hospital, Benevento*; M. De Mari, C. Diroma, G. Iliceto, P. Lamberti, *University of Bari*; V. Toni, G. Trianni, *Hospital of Casarano*; A. Mauro, *Hospital of Salerno*; A. De Gaetano, *Hospital of Castrovillari*; M. Rizzo, *Hospital of Palermo*.

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ELECTRONIC LETTER

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

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J Med Genet 2005;42:e (<http://www.jmedgenet.com/cgi/content/full/42/11/e65>). doi: 10.1136/jmg.2005.035568

Background: Mutations in the gene *Leucine-Rich Repeat Kinase 2 (LRRK2)* were recently identified as the cause of PARK8 linked autosomal dominant Parkinson's disease.

Objective: To study recurrent *LRRK2* mutations in a large sample of patients from Italy, including early (<50 years) and late onset familial and sporadic Parkinson's disease.

Results: Among 629 probands, 13 (2.1%) were heterozygous carriers of the G2019S mutation. The mutation frequency was higher among familial (5.1%, 9/177) than among sporadic probands (0.9%, 4/452) ($p<0.002$), and highest among probands with one affected parent (8.7%, 6/69) ($p<0.001$). There was no difference in the frequency of the G2019S mutation in probands with early v late onset disease. Among 600 probands, one heterozygous R1441C but no R1441G or Y1699C mutations were detected. None of the four mutations was found in Italian controls. Haplotype analysis in families from five countries suggested that the G2019S mutation originated from a single ancient founder. The G2019S mutation was associated with the classical Parkinson's disease phenotype and a broad range of onset age (34 to 73 years).

Conclusions: G2019S is the most common genetic determinant of Parkinson's disease identified so far. It is especially frequent among cases with familial Parkinson's disease of both early and late onset, but less common among sporadic cases. These findings have important implications for diagnosis and genetic counselling in Parkinson's disease.

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

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

An autosomal dominant form of Parkinson's disease (PARK8) was first mapped to chromosome 12 in a Japanese family⁴; this linkage was later confirmed in white families.^{5–6} Recently, mutations in the gene *Leucine-Rich Repeat*

Kinase 2 (LRRK2) (MIM *609007) were identified in PARK8 linked families.^{7–8} The *LRRK2* gene encodes a predicted protein of 2527 amino acids, which has unknown function. This protein, termed dardarin, belongs to the ROCO group within the Ras/GTPase superfamily, and contains several conserved domains: an Roc (Ras in complex proteins) and a COR (C-terminal of Roc) domain, together with a leucine-rich repeat, a WD40 domain, and a tyrosine kinase catalytic domain.⁹

To date, seven *LRRK2* pathogenic mutations have been reported in autosomal dominant Parkinson's disease. Four of these mutations occurred in at least two unrelated families: Y1699C (present in two large kindreds, family "A" of German-Canadian ancestry, and one British kindred)^{7–8}; R1441C (found in family "D" of Western Nebraska origin, and another family)⁶; R1441G (found in several families and a few sporadic cases in the Basque population)⁷; and G2019S, which we and other groups have recently identified.^{10–14}

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

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

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

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

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

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

METHODS

Subjects and clinical analyses

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

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

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

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

Mutation analysis

Genomic DNA was isolated from peripheral blood using standard protocols.¹⁶ The primers and polymerase chain reaction (PCR) protocol used to amplify the *LRKK2* exons (Nos 31, 35, and 41) containing the C4321T (R1441C), C4321G (R1441G), the A5096G (Y1699C), and the G6055A (G2019S) mutation have been reported previously.¹⁰ The consequences of mutations at the protein level were predicted according to the *LRKK2* cDNA sequence (Genbank accession number AY792511).

About 20 ng of pooled PCR product (exons 31, 35, and 41) were purified using ExoSAP-IT (USB) and used in a primer extension reaction (SNaPshot) including the following

primers: for the R1441C and the R1441G mutations in exon 31 (sense strand), 5'-agaatcacagggaagaagaagcgc-3', product size 26 base pairs (bp) (primer length plus one base); for the Y1699C mutation in exon 35 (antisense strand), 5'-taatc-gattgattaatcttgacaaaatccattggaaaa-3', product size 41 bp; for the G2019S mutation in exon 41 (antisense strand), 5'-aatgctgccatcattgcaaagattgctgactac-3', product size 34 bp.

Reactions were carried out in 10 μ l containing 1 μ l SNaPshot multiplex ready reaction mix (Applied Biosystems, Foster City, California, USA); 2.5 μ M R1441C/R1441G, 7.5 μ M Y1699C, 2.5 μ M G2019S extension primer, and 1 μ l $\frac{1}{2}$ term buffer (200 mM TrisHCl; 5 mM MgCl₂, pH 9). Additional thermal cycling was undertaken for 40 cycles of 10 seconds at 95°C, five seconds at 50°C, and 30 seconds at 60°C. Removal of the 5'-phosphoryl groups was done using 1 unit of shrimp alkaline phosphatase (SAP) (Roche Diagnostics, Monza, Italy) for 30 minutes at 37°C.

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

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

In one case carrying the G2019S mutation and one control, total RNA was isolated from blood cells and cDNA was prepared using standard protocols. A 251 bp fragment of the *LRKK2* cDNA spanning exons 41–42 was amplified using the following primers: forward 5'-cacgtagctgatgtgttgatagacc-3'; reverse 5'-ccaatgaataaacatcagcctgt-3'.

Haplotype analysis

Nineteen intragenic and flanking markers (13 microsatellites and six single nucleotide polymorphisms (SNP)) were typed, including both known exonic and a newly discovered *LRKK2* intronic SNP (IVS13+104G/A) in linkage disequilibrium (LD) with the G2019S mutation. Microsatellites were selected from the Marshfield integrated map and from Kachergus *et al*¹⁴; they were amplified by PCR using fluorescently labelled F-primers according to standard methods; fragments were loaded on an ABI3100 and analysed using the GeneMapper version 3.0 software (Applied Biosystems). Exonic and intronic *LRKK2* SNPs were typed by direct sequencing using the primers and PCR conditions reported previously.¹⁰

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

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

Table 2 Frequency of the G2019S mutation according to familial aggregation

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

*p<0.002 v the frequency among the 452 sporadic probands (Fisher exact test).

†p<0.025; frequency in familial v sporadic early onset probands.

‡p=0.05; frequency in familial v sporadic late onset probands.

§p<0.001 v the frequency among the 452 sporadic probands.

NSNo significant difference compared with the frequency among the 452 sporadic probands.

None of the differences between early onset and late onset groups (familial, sporadic, or all) was statistically significant.

PD, Parkinson's disease.

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

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

respectively) identified by us from unrelated series of patients. Haplotypes were constructed manually. In four families phase could be assigned unambiguously for most markers by genotyping of trios of parents and child. In the remaining families, the phase was estimated using PHASE version 2.1.¹⁷ Haplotypes with known phase were included to improve the performance of the program. Statistical analysis was undertaken using contingency tables and the Student's *t* test, as appropriate.

RESULTS

Frequency of mutations

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

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

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

Among 600 probands tested, there was one heterozygous for the R1441C mutation but none carrying the R1441G or

the Y1699C mutation. These mutations were not observed in controls.

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

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

Haplotype analysis

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

Clinical features

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

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

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

Frequency of *LRRK2* mutations in Parkinson's disease

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

Origin of the G2019S mutation from a common founder

Our haplotype analysis strongly suggests that the G2019S mutation is transmitted from a single ancient founder. This confirms the results of a previous study,¹⁴ and refines the size of the shared region on the 3' end of the *LRKK2* gene, excluding markers D12S2519 and D12S2520. More importantly, in our data the ~160 kb minimum shared region spans the promoter and most of the *LRKK2* gene, suggesting that variation at the promoter or other *cis*-acting regulatory elements are not important determinants of the phenotypic variation observed among G2019S carriers. However, variants at regulatory elements in the other allele might play a modifier role. In the previous study the minimum shared region was reduced to 145 kb from marker D12S2515 to D12S2521, thereby excluding the promoter and the first 21 exons and 20 introns of *LRKK2*.¹⁴ However, our data suggest that D12S2515 is a highly unstable microsatellite, and the observed data in this study and the previous study¹⁴ are also compatible with mutations occurring in this

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

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

Values are mean (SD).

†Years from the age at onset of symptoms to the age at last examination.

* $p < 0.02$ v G2019S het. male carriers (Student's *t* test).

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

The clinical phenotype associated with G2019S

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

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

earlier in female carriers. Further studies are also needed to assess prospectively the rate of progression of the disease associated with this and other *LRRK2* mutations.

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

Conclusions

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

ACKNOWLEDGEMENTS

We thank all the patients and family relatives for their contribution, Dr Francesca Sciacca, National Neurological Institute "C Besta", Milan, Italy, for providing some of the control samples, and Tom de Vries-Lentsch, Erasmus MC Rotterdam, for artwork. The DNA samples contributed by the Parkinson Institute – Istituti Clinici di Perfezionamento, Milan, Italy, were from the "Human genetic bank of patients affected by Parkinson disease and parkinsonisms", supported by Telethon grant No GTF03009. The study was supported by grants from the Internationaal Parkinson Fonds (Netherlands) and the National Parkinson Foundation (USA) to VB.

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Competing interests: none declared

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Received 3 June 2005

Revised version received 22 June 2005

Accepted for publication 27 June 2005

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Early-onset Parkinson's disease caused by a novel *parkin* mutation in a genetic isolate from north-eastern Brazil

Received: 18 July 2005 / Accepted: 30 August 2005 / Published online: 22 November 2005
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Abstract We describe clinical and molecular findings in a genetic isolate from north-eastern Brazil with early-onset Parkinson's disease (PD) and a novel mutation in the *parkin* gene. Genealogical studies could connect 255 individuals, of whom 15 had PD. Geographic isolation and multiple consanguineous marriages initially suggested an autosomal recessive inheritance for PD in these patients. The available individuals were personally examined, and DNA was obtained from 26 members: ten early-onset PD patients, one case with likely neuroleptic-induced parkinsonism and 15 unaffected relatives. The average age at onset of PD symptoms was 30.8 years (range 12–46). Haplotype analysis revealed homozygosity in the PD patients for markers across the PARK2 locus. Genomic sequencing identified a novel homozygous splice-site *parkin* mutation (IVS1+1G/T), which completely co-segregated with the early-onset PD phenotype. cDNA analysis confirmed the total loss of *parkin* transcript in homozygous mutation carriers, delineating this as a loss-of-function mutation. The case with neuroleptic-induced parkinsonism and 13 of 15 healthy relatives were heterozygous carriers of the mutation. The absence of PD in heterozygous carriers indicates a gen-

uinely recessive nature of this mutation, suggesting that *parkin* haploinsufficiency is not a relevant risk factor for early- or late-onset PD. However, *parkin* haploinsufficiency could facilitate the emergence of neuroleptic-induced parkinsonism. The cluster reported here, which to our knowledge is the largest described to date with early-onset PD and *parkin* mutations, also offers a unique opportunity for the search of modifiers of the *parkin*-related disease.

Keywords Parkinson disease · *parkin* · Gene · Mutation · Genetic isolate

Introduction

In recent years, family-based linkage mapping and positional cloning studies have led to the identification of several mendelian forms of Parkinson's disease (PD) (MIM #168600), including autosomal dominant and recessive forms [1]. Mutations in the *parkin* gene (MIM*602544) are the most common known cause of autosomal recessive, early-onset PD, being found in about half of the families with early-onset PD compatible with recessive inheritance and in about 10–15% of the isolated early-onset cases (early-onset defined in most studies as the onset of symptoms before the age of 45 years) [2–4].

Although several mutations have been described worldwide, different aspects of the PD form caused by *parkin* mutations (the *parkin*-related disease) remain poorly understood. The protein encoded by *parkin* has ubiquitin ligase activity [5–7], and it interacts with the proteasome, [8–10] suggesting a role in protein degradation pathways. However, the mechanism of the disease caused by *parkin* mutation remains mostly unknown. From the genetic standpoint, in several studies, despite comprehensive screening, a single heterozygous mutation has been found in few patients with early-onset PD [3, 4, 11, 12], suggesting that some *parkin* mutations can be pathogenic in single heterozygous state. Other studies have suggested that carrying a single heterozygous mutation in this gene is a risk factor for late-onset PD [13, 14]. Last, a wide variability of onset ages is

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observed in patients with *parkin*-related disease, even in single families [15], suggesting the existence of modifiers of the phenotype.

Here we describe clinical and molecular findings in an extended family from an isolated region in north-eastern Brazil with early-onset PD and a novel splice site *parkin* mutation. To our knowledge, this is the largest cluster of PD cases due to *parkin* mutations so far reported, offering unique opportunities for studying the genetic and clinical aspects of this form of human neurodegenerative disease.

Methods

Clinical and genealogical studies

A neurologist with experience in movement disorders (H.F.C.) examined all available members of the family. Genealogical investigations were based mainly on the information provided by the living family members. The diagnosis of clinically definite PD was established according to widely accepted criteria [16] and required the presence of bradykinesia and at least one of the following: resting tremor, rigidity and postural instability; a positive response to dopaminergic therapy; the absence of atypical features or other causes of parkinsonism. The diagnosis of clinically likely PD was made when the clinical features were those typical for PD but a response to dopaminergic therapy was not documented. Neurological examination included the Unified Parkinson's Disease Rating Scale (UPDRS, motor part) [17] and Hoehn–Yahr staging. The project was approved by the relevant ethical authorities, and written informed consent was obtained from all subjects.

Genetic studies

All available affected and unaffected family members aged ≥ 18 years were included. Genomic DNA was isolated from peripheral blood using standard protocols. For haplotype analysis, we typed short tandem repeat (STR) markers from the PARK2 (*parkin*), PARK6 (*PINK1*) and PARK7 (*DJ-1*) regions as previously reported [18] using fluorescently labelled primers, according to standard methods; fragments were loaded on an ABI3100 and analysed using the GeneMapper ver. 3.0 software (Applied Biosystems). Haplotypes were constructed based on the minimum number of recombinations.

DNA fragments covering exons 2–12 and splice sites of the *parkin* gene were amplified according to our polymerase chain reaction (PCR) protocol detailed elsewhere [19]. *Parkin* exon 1 was amplified in 20 μ l containing 1 $\times$ GCII TaKaRa buffer, 400 μ M of each dNTP, 1 μ M forward primer, 1 μ M reverse primer, 1 unit of LA *Taq* DNA polymerase (TaKaRa) and 25 ng genomic DNA. Cycle conditions were: 7 min 30 s at 96°C; 35 cycles of 30 s denaturation at 96°C, 30 s annealing at 68°C, 1 min 30 s extension at 72°C; final

extension 5 min at 72°C. The following primers were used to amplify *parkin* exon 1: 5'-ctgggggcaggagcgtag-3' (forward), and 5'-ggacggcacgggcactttgg-3' (reverse), product size 357 bp.

Total RNA was isolated from blood cells, and cDNA was prepared using reverse transcriptase PCR (RT-PCR) standard protocols from two homozygous and one heterozygous carrier of the *parkin* IVS1 + 1G/T mutation and from several unrelated controls. Two fragments of the *parkin* cDNA (Genbank accession number NM_004562) spanning exons 1–3 and 4–6 were amplified using a touchdown PCR protocol and the following primers: (exons 1–3) 5'-aggagaccgctgggtggag-3' (forward) and 5'-ccacctccttgagctggaag-3' (reverse), product size 173 bp; (exons 4–6) 5'-gtcaaagagtcagccggg-3' (forward) and 5'-ctattgttcgatcaggtgc-3' (reverse), product size 238 bp. A fragment of the *hypoxanthine phosphoribosyltransferase-1* (*HPRT*) cDNA was also amplified as a control transcript, using the following primers: 5'-cgtgggtcctttcaccagcaag-3' (forward) and 5'-aattatggacaggactgaacgtc-3' (reverse), product size 385 bp.

PCR products were purified using 2 μ l ExoSAP-IT (USB) for 30 min at 37°C, followed by a 10-min inactivation step at 80°C. Direct sequencing of both strands was performed using Big Dye Terminator chemistry ver. 3.1 (Applied Biosystems). Fragments were loaded on an ABI3100 automated sequencer and analysed with DNA Sequencing Analysis (ver. 3.7) and SeqScape (ver. 2.1) software (Applied Biosystems).

Results

Genealogical studies

The ancestors of the family were of white ethnicity and Portuguese origin, and they established a small settlement in the state of Paraíba, Brazil, in 1860. Because of the geographical isolation and the local traditions, the family has had the practice of consanguineous marriage since then. The descendants are today distributed in four clusters living in different states of Brazil, although the biggest branch of the family is still residing in Paraíba. The pedigree is depicted in Fig. 1. So far, 255 individuals belonging to the kindred could be linked genealogically. Fifteen persons are known to have PD. Of them, ten (seven males and three females) have been personally examined by H.F.C., and five of them are regularly followed at the Department of Neurology, University of São Paulo.

Clinical studies

Twenty-six members of the family were examined personally by one of the authors (H.F.C.). Of these 26 individuals, ten received a clinical diagnosis of PD: nine had clinically definite PD, and one had likely PD (this last case was not yet treated with levodopa, and therefore, a good response to this

Fig. 1 Pedigree of the family. *Black symbols* denote individuals affected by PD, and a *question mark within symbol* indicates the individual with likely neuroleptic-induced parkinsonism. Individual codes are reported below symbols for all persons with available *parkin* genotype. *HOM* homozygous IVS1+1G/T mutation, *Het*

heterozygous IVS1+1G/T mutation, *WT* wild-type genotype (IVS1+1G/G). The age at examination of the 13 unaffected heterozygous mutation carriers was as follows (subject #, years): #73, 62; #101, 66; #111, 69; #123, 82; #149, 60; #161, 32; #176, 79; #181, 67; #198, 44; #215, 49; #223, 52; #242, 24; #245, 18

drug could not be documented). Detailed clinical findings in these ten PD cases are reported in Table 1. The average age at examination of PD patients was 45.1 years (range 30–67). The average age at onset of PD symptoms was 30.8 years. A wide range of onset ages is observed, from 12 to 46 years. However, most cases had onset between the age of 30 and 38 years. In only two cases, onset occurred before the age of 20 (#96, the index case, onset age 14; and #193, onset age 12), and in only one case was the onset after the age of 40 (#95, onset age 46). Patient #150 displayed the highest UPDRS motor scores, which can be explained, at least in

part, by the very long disease duration and by the fact that she could only tolerate small doses of levodopa because of drug-induced side effects (disabling dyskinesias).

Another individual (#210), aged 41 at the time of the examination, had had bilateral arm tremor and bradykinesia since the age of 38. She presented hallucinations and other psychiatric disturbances around age 37, for which she was treated with medications including neuroleptics, and she was still on neuroleptic therapy (haloperidol) at the time of our examination; a diagnosis of likely neuroleptic-induced parkinsonism was therefore made for this case.

Table 1 Clinical features in ten PD patients with homozygous IVS1+1G/T mutation

Subject code	Onset age (year)	Disease duration (year)	Asymmetric onset	Bradykinesia	Tremor	Rigidity	Dystonia	UPDRS (on)	H-Y (on)	L-dopa therapy	L-dopa dose (mg)	Fluct/dysk	CT/MRI
95	46	7	+	+	+	–	–	14	2.5	+	625	+	Normal
96	14	24	+	+	+	+	–	14	3	+	500	+	Normal
104	34	34	+	+	+	+	–	44	4	+	250	+	Mild diffuse atrophy
142	38	7	+	+	+	+	–	43	3	+	300	+	NA
150	36	24	+	+	+	+	+	100	5	+	250	+	NA
162	30	11	+	+	+	+	–	29	3	+	500	+	NA
163	35	4	+	+	+	+	–	20	2.5	–	–	–	NA
165	30	6	+	+	+	+	–	29	2.5	+	375	–	NA
193	12	18	+	+	+	+	–	20	2	+	500	+	Normal
199	33	8	+	+	+	+	–	31	3	+	375	–	Normal

The plus sign (+) indicates the presence of the feature; the minus sign (–) indicates its absence

UPDRS Unified Parkinson's Disease Rating Scale, H-Y Hoehn-Yahr staging, Fluct/dysk levodopa-induced motor fluctuations and dyskinesias, CT brain computed tomography, MRI brain magnetic resonance imaging, NA not performed

Apart from individual #210, none of the patients suffered from psychiatric or behavioural disturbances and none had dementia. The 15 remaining members were free from PD symptoms and signs.

Genetic studies

Haplotype analysis revealed that PD patients were homozygous for markers across the PARK2 locus (on chromosome 6q25-q27) (Fig. 2a), but not the PARK6 and PARK7 loci (data not shown), supporting the involvement of the *parkin* gene. Sequencing the 12 exons and splice sites of *parkin* in the proband revealed a novel homozygous mutation in the splice donor site of exon 1 (IVS1+1G/T, Fig. 2b–d). No other sequence changes were detected.

All of the 10 patients diagnosed with PD were homozygous carriers of the IVS1+1G/T mutation. The individual with neuroleptic-induced parkinsonism and 13 of the 15 unaffected relatives were heterozygous carriers of the mutation. The remaining two unaffected individuals did not carry the mutation. Thus, there was complete co-

segregation between the PD phenotype and the homozygous IVS1+1G/T genotype.

The average age at last examination for the 13 unaffected relatives who were heterozygous carriers of the IVS1+1G/T mutation was 54.2 years (range 18–82); only four of them were younger than 46 years, the oldest age at onset of PD observed in this family.

In both PD patients from whom mRNA from blood cells was available, we could not amplify any of the two fragments of the *parkin* cDNA (across exons 1–3 and exons 4–6), whereas the amplification of a band of the expected size was obtained from the unaffected heterozygous carrier of the IVS1+1G/T mutation and from several unrelated controls (Fig. 2f,g). The PCR products were sequenced to confirm accuracy of *parkin* cDNA amplification (data not shown). These results are in line with the expected lack of *parkin* mature mRNA in the patients carrying the splice site mutation in homozygous state.

The cDNA from control genes, such as *HPRT* (Fig. 2e) and other genes (data not shown), could be normally amplified from all the individuals analysed (patients and unaffected), indicating that the abnormality detected in the PD patients was specific for the *parkin* cDNA.

Fig. 2 Genetic findings. **a** Haplotypes of the PARK2 locus in five representative individuals. Markers are ordered according to the Marshfield integrated map. Two *parkin* intragenic markers are highlighted in blue. **b–d** Representative electropherograms of part of the *parkin* exon 1, showing the IVS1+1G/T mutation (arrow) in homozygous (**b**) and heterozygous (**c**) state and the wild-type genotype (**d**). **e–g** RT-PCR analysis of a control (*HPRT*) (**e**) and the *parkin* (**f**, **g**) transcript, showing the selective absence of *parkin* transcript in the patients. **C** unrelated control individuals, **red arrowhead** two PD patients homozygous carriers of the IVS1+1G/T mutation, **grey arrowhead** unaffected relative, heterozygous carrier of the IVS1+1G/T mutation.

Discussion

The findings of this study are relevant for several reasons. To our knowledge, this is the largest kindred with early-onset PD due to *parkin* gene mutations; the PD-causing mutation in this family is novel, and one of the very few splice site mutations reported so far in this gene.

The mutation is predicted to disrupt the splicing of the *parkin* mRNA because it affects the conserved splice donor of exon 1; the presence of this mutation is predicted to lead to the formation of an aberrantly long mRNA species, which is unstable and rapidly degraded. The results of the RT-PCR analysis are in line with the expected absence of mature *parkin* mRNA in the patients, who are homozygous carriers of the splice site mutation.

A different pathogenic mutation at the very same nucleotide position (IVS1+1G/A) was previously reported in compound heterozygosity with a frame-shift mutation (c.202–203delAG in exon 2) in a Russian family with autosomal recessive, early-onset PD (onset age 17 and 23 years) [20]. mRNA studies were not performed in that family. A recent review lists 95 *parkin* mutations identified in PD patients, including 40 exon rearrangements and 55 point mutations or small deletions or insertions [4]. Remarkably, only four of these 95 mutations affect splice sites: IVS1+1G/A [20], IVS5+2T/A [12], IVS7-1G/C [12] and IVS9+4G/T [14]; we recently reported a fifth splice site mutation in a Cuban family (IVS11-3C/G) [19].

By cDNA analysis from patients' material, we functionally characterized the IVS1+1G/T mutation as a loss-of-function, pathogenic mutation; the assessment of the functional effect of novel variants detected in PD-related genes has been performed rarely; yet, this is important to allow accurate genotype–phenotype correlation studies to be performed and to orient the genetic counselling.

By analysing several affected and unaffected members of the family, we showed the complete co-segregation of the mutation in homozygous state with PD and the absence of PD in heterozygous carriers. These findings confirm the disease-causing role of the IVS1+1G/T mutation and delineate it as a classical loss-of-function, recessive mutation. According to our results, being a heterozygous carrier of a loss-of-function allele (haploinsufficiency) of *parkin* does not seem to be a major risk factor for developing early- or late-onset PD, unless a different genetic or environmental trigger is also present, such as the neuroleptic exposure in one family member reported here.

In fact, one of the heterozygous carriers of the IVS1+1G/T mutation (#210) showed signs of parkinsonism after neuroleptic exposure. Interestingly, she is the only patient in the family with a bilateral onset of symptoms, which also suggests an etiology different than that in her relatives. In the context of the exposure to neuroleptics, the most likely diagnosis is drug-induced parkinsonism. However, it is conceivable that the haploinsufficiency of the *parkin* gene acted in this person as a predisposing factor for the development of parkinsonism after neuroleptic exposure. Ac-

cordingly, previous PET studies documented mild, sub-clinical dopaminergic dysfunction in asymptomatic heterozygous carriers of *parkin* mutations [21, 22]. A further follow-up might clarify if the parkinsonism in individual #210 will persist after withdrawal of neuroleptics or not.

The observations in the family reported here do not exclude that other *parkin* mutations might cause disease in single heterozygous state. This might be particularly true for mutations, which act through a dominant or a dominant-negative effect. The R275W mutation has been associated most frequently with disease in single heterozygous state [4]. Recently, it has been reported that *parkin* protein bearing missense mutations such as R275W form aggregates in cell cultures and might therefore also have toxic, gain-of-function properties [23, 24].

In keeping with the phenotype reported in most patients with *parkin* mutations [15], the clinical phenotype in ten PD patients with homozygous IVS1+1G/T mutation is characterized by parkinsonism of early-onset, slow progression, and by occurrence of levodopa-induced motor fluctuations and dyskinesias. Interestingly, a wide variability of onset age is evident, from 12 to 46 years, suggesting the presence of strong modifiers of the phenotype caused by *parkin* mutation.

The mild scores of the UPDRS in several patients after many years from the disease onset are a clear, albeit indirect, evidence of a slow clinical progression of the disease. The development of motor fluctuations and choreic involuntary movements (dyskinesias) after years of levodopa therapy is considered a response of the dopamine-depleted striatum to the long-term non-physiological dopaminergic stimulation (levodopa) [25]. This is a general feature in PD and is considered a confirmatory criterion for the diagnosis of “idiopathic” degenerative PD [16].

Other features frequently associated with *parkin* mutation, such as bilateral onset of symptoms, dystonic features at onset, amelioration of symptoms after sleep (sleep benefit), psychiatric and behavioural disturbances [15], were not present in our family. Dementia is rare in patients with *parkin* mutations, and it was not present in the family described here.

The onset of PD symptoms was asymmetric in all patients with homozygous *parkin* mutation. Only in the person with neuroleptic exposure did parkinsonism signs started bilaterally, once again pointing to a different disease etiology.

In contrast with the remarkable etiologic heterogeneity reported in another large PD family with *parkin* mutations in some of the affected members [26], a homogeneous genetic etiology characterizes PD in the family cluster described here.

Due to the exceptional size, this family offers a unique opportunity to search for genetic and non-genetic modifiers of the *parkin*-related disease. Candidate modifier genes include the other PD-causing genes, particularly those causing autosomal recessive forms (*PINK1* and *DJ-1*) [27, 28], as well as genes emerging from systematic screens for

modifiers of the phenotype induced by *parkin* mutation in *Drosophila* [29].

Acknowledgements We thank the members of the family for their contribution to this study and Tom de Vries-Lentsch for artwork. This work was supported by grants from the National Parkinson Foundation (USA) to V. Bonifati and from CAPES (Brazil) to H.F. Chien.

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

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GLOSSARY

PHOTOCHEMICAL
INTERNALIZATION (PCI)

Using photoirradiation and a hydrophilic photosensitizer to enhance the delivery of endocytosed plasmid DNA to the cytoplasm

VENUS REPORTER GENE

A variant of yellow fluorescent protein that can be used to detect cells that express the transfected plasmid DNA

***In vivo* gene transfer by delivery of DNA packaged in a dendrimeric photosensitizer**

Synthetic vectors made of cationic peptides combined with plasmid DNA (pDNA) might enable safer, better targeted and more-efficient means of delivering genes to cells *in vivo* than 'traditional' viral methods. Nishiyama and co-workers developed a targeted method to deliver transgene-containing pDNA, in a complex with positively charged peptides that contain a nuclear localization signal, using light-sensitive chemicals incorporated into a synthetic vector. Following endocytosis of these complexes, the integrity of the endosomal membrane can be further disrupted by photoirradiation, thus giving the plasmid greater access to the nucleus. In their investigation, Nishiyama *et al.* tested whether PHOTOCHEMICAL INTERNALIZATION (PCI) could be used to spatially control transfection *in vivo* through photoirradiation of target sites.

In vitro experiments demonstrated that photosensitization of cells that had endocytosed the vector resulted in increased release of endosomal contents into the cytoplasm, giving pDNA increased access to the nucleus. In addition, PCI increased expression of the transgene over 100-fold, with minimal cytotoxic effects. *In vivo* experiments using a VENUS REPORTER GENE showed that injection of the pDNA complex into subconjunctival tissue of rats' eyes, followed by laser irradiation, resulted in gene expression in the irradiated site in 8/12 eyes.

This is the first report of successful PCI-mediated gene transfer *in vivo*. PCI holds promise for improving drug delivery and gene transfer for the treatment of many diseases, including solid tumors and ophthalmic disease. Spatial control of gene delivery through localized laser treatment should enhance the effectiveness and safety of the procedure.

Kate Matthews

Original article Nishiyama N *et al.* (2005) Light-induced gene transfer from packaged DNA enveloped in a dendrimeric photosensitizer. *Nat Mat* 4: 934–941

Can seizure remission indicate outcome in PNESs?

Psychogenic nonepileptic seizures (PNESs) are paroxysmal episodes that are commonly misdiagnosed as epilepsy. Misdiagnosis can

lead to inappropriate treatment being administered, which can have serious implications. A high proportion of PNES patients become unemployed as a result of their illness and are financially supported through government benefit schemes. These factors have stimulated research into effective treatments, but the psychosocial problems experienced by patients may mean that traditional outcome assessments (based on cessation of seizures) are not relevant. Reuber *et al.* investigated whether seizure remission indicates outcome in PNES patients as it does for epileptic patients.

A questionnaire was sent to the 329 PNES patients who had been diagnosed at Bonn University, Germany between April 1991 and April 2001. Patients were asked to answer questions (from widely used, clinically validated questionnaires) that led to a current psychopathology score and a somatization score. Questionnaires were returned by 164 patients; of the 147 who answered the questions completely enough to be included in analyses, 61 had concurrent epilepsy. Clinical demographics were similar for responders and nonresponders.

Seizures continued in 105/147 patients, but seizure-free status was not a statistically or clinically significant indicator of financial productivity: only 50% of those without seizures had better quality of life and nearly half were unproductive or had continuing psychosocial problems. Only higher educational achievement and younger age at time of the study were statistically significant indicators of seizure-free, financially productive status ($P=0.020$ and $P=0.039$, respectively).

The authors conclude that seizure remission is an unreliable measure of clinical and psychosocial outcome in patients who have experienced PNESs. Controlling seizures should therefore not be the only focus of PNES treatment.

Rebecca Ireland

Original article Reuber M *et al.* (2005) Measuring outcome in psychogenic nonepileptic seizures: how relevant is seizure remission? *Epilepsia* 46: 1788–1795

A novel *parkin* mutation is linked with early-onset PD

Mutations in the *parkin* gene are the most commonly identified cause of autosomal

recessive, early-onset Parkinson's disease (PD). To improve understanding of the intricacies of this severe Mendelian form of PD, a team from Sao Paulo University, Brazil, and Erasmus MC Rotterdam, The Netherlands, studied what they believe to be the largest reported cluster of early-onset PD cases resulting from *parkin* mutations, in an extended family of 255 from an isolated region in Brazil.

Of the 26 individuals from whom DNA was obtained, 10 had early-onset PD and 1 had probable neuroleptic-induced parkinsonism; the remaining 15 showed no signs or symptoms of PD. Although most cases experienced PD symptom onset between the ages of 30 and 38 years, the wide range of ages at onset (12–46 years) suggests the presence of strong modifiers of the disease phenotype. Disease duration ranged from 6 to 34 years, and was characterized by slow clinical progression, and by motor fluctuations and dyskinesias caused by levodopa therapy. In the 10 PD patients, both copies of the *PARK2* locus had the same gene markers and splice-site mutation (homozygosity); total loss of the *parkin* transcript was confirmed by cDNA analysis. Of the remaining 16 individuals, 14 (including the one with neuroleptic-induced parkinsonism) had only one copy of the mutation (heterozygosity).

The authors therefore confirm that a “classical loss-of-function, recessive mutation” has a role in this disease form. Heterozygosity of the *parkin* mutation would not appear to be a major risk factor for PD development, without an additional factor being present.

Pippa Murdie

Original article Chien HF *et al.* (2005) Early-onset Parkinson's disease caused by a novel *parkin* mutation in a genetic isolate from north-eastern Brazil. *Neurogenetics* [doi: 10.1007/s10048-005-0017-x]

Hippocampal volume and shape can help predict response to donepezil treatment

Results of a recent neuroanatomical study of patients with dementia of the Alzheimer type (DAT) indicate that certain variations in hippocampal structure and volume correlate with a poorer response to treatment with donepezil, an acetylcholinesterase inhibitor.

Csernansky and colleagues obtained baseline hippocampal MRI images for 37 patients (mean age 74.8 years) with mild DAT who were commencing treatment with donepezil 10 mg daily. They analyzed the images using brain-mapping algorithms to determine hippocampal volume and shape. Growth curve models were used to estimate the rates of change in patient clinical status—patients were assessed every 3 months over a 2-year period using the cognitive subscale of the Alzheimer's Disease Assessment Scale (ADAS-cog).

The researchers found a significant correlation between inward variation of the inferomedial zone (IMZ) and lateral zone (LZ) of the hippocampal surface and more rapid worsening of ADAS-cog scores ($P=0.02$ for left IMZ; $P=0.05$ for right IMZ; $P=0.09$ for left LZ; $P=0.02$ for right LZ). Smaller left and right hippocampal volumes also correlated with more rapid worsening of ADAS-cog scores ($P=0.04$ and $P=0.02$, respectively).

The authors suggest that, although the magnitude of the correlations observed was not large enough to explain all the variance in ADAS-cog scores, neuroanatomical measures such as those used in their study might be useful in helping to preselect patients with DAT for whom treatment with acetylcholinesterase inhibitors would be beneficial.

Christine Kyme

Original article Csernansky JG *et al.* (2005) Neuroanatomical predictors of response to donepezil therapy in patients with dementia. *Arch Neurol* 62: 1718–1722

Lipid-lowering agents and cognitive decline in AD

An observational study was recently carried out in France in which researchers investigated whether use of lipid-lowering agents (LLAs) was associated with a slower rate of cognitive decline in patients with Alzheimer's disease (AD).

Masse and colleagues followed 342 AD patients (232 female, 110 male; mean age 73.5 years) for a mean of 34.8 months—129 patients were dyslipidemic and were being treated with LLAs (47% with statins), 105 were dyslipidemic but not receiving LLAs, and the remaining 108 patients were normolipidemic. Baseline Mini-Mental State Examination

ARTICLE

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

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

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²⁷Members are listed in the Appendix

Received 9 September 2005; revised 14 October 2005; accepted 18 October 2005; published online 7 December 2005

p.I1371V mutation, for which a pathogenic role could not be established with certainty. In total, 13 novel disease-unrelated variants and three intronic changes of uncertain significance were also characterized. The phenotype associated with *LRRK2* pathogenic mutations is the one of typical PD, but with a broad range of onset ages (mean 55.2, range 38–68 years) and, in some cases, slow disease progression. On the basis of the comprehensive study in a large sample, we conclude that pathogenic *LRRK2* mutations are frequent in ADPD, and they cluster in the C-terminal half of the encoded protein. These data have implications both for understanding the molecular mechanisms of PD, and for directing the genetic screening in clinical practice.

European Journal of Human Genetics (2006) **14**, 322–331. doi:10.1038/sj.ejhg.5201539; published online 7 December 2005

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

Introduction

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

A different locus, PARK8 (MIM #607060), was first mapped to chromosome 12 in a Japanese family with dominantly inherited parkinsonism.⁸ Recently, mutations in the gene *leucine-rich repeat kinase 2* (*LRRK2*) (MIM *609007) have been identified in PARK8-linked families.^{9,10} The *LRRK2* gene encodes a predicted protein of 2527 amino acids, which has an unknown function. The *LRRK2* protein, also termed dardarin, belongs to the ROCO group within the Ras/GTPase superfamily, characterized by the presence of several conserved domains: a Roc (Ras complex proteins) and a COR (C-terminal of Roc) domain, together with a leucine-rich repeat region, a WD40 domain, and a protein kinase catalytic domain.¹¹

To date, five *LRRK2* missense mutations associated with autosomal dominant PD (p.R1441C, p.R1441G, p.Y1699C, p.G2019S, and p.I2020T)^{9,10,12–15} are considered definitely pathogenic on the basis of clear cosegregation with disease in large pedigrees and absence in controls. The evidence for cosegregation with PD is limited for another two mutations found in small families (p.L1114L and p.I1122V),^{9,16} whereas it is lacking for four additional mutations because DNA from relatives was unavailable (p.I1371V and p.R1441H),^{17,18} or because the mutation was identified in single sporadic PD cases (IVS31+3A>G and

p.M1869T);^{16,17} the pathogenic role of these last six mutations remains therefore uncertain.

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

Materials and methods

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

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

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

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

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

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

Results

Genetic studies

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

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

Initial results concerning the four families with the p.G2019S mutation have been previously published by

us,¹² whereas the other three families with *LRRK2* mutations as well as the results of the comprehensive analysis of the *LRRK2* gene in the entire sample of 60 ADPD probands are reported here for the first time.

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

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

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

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

Clinical studies

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

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

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

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

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

Discussion

Frequency and nature of *LRRK2* mutations

To our knowledge, this is the first study which comprehensively analysed all the 51 exons and the exon-intron boundaries of the *LRRK2* gene in a large sample of 60

ADPD probands (mostly from Italy), revealing the presence of two recurrent pathogenic mutations, p. G2019S and p.R1441C, in six families (10% of the whole sample, 8% of the Italian sample), and a third mutation, p.I1371V, in another family. These frequencies are in substantial agreement with those reported in the only two previous studies of comparable size, which comprehensively screened the *LRRK2* gene, and found mutations in 3/23 and 6/34 families, respectively (13% and 17%).^{9,18} ADPD represents a relevant fraction of the whole population of PD. According to the results of this

b

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

d

Species	Position	Sequence	Position	Sequence
homo sapiens	1358-	KKSDLGMQSA TVG	1385	DVKDWP IQIRDKRK - 1385
bos taurus	107-	KKSDLGMQGA TVG	134	DVKDWP IQIRGK GK - 134
rattus norvegicus	1370-	KKSE LGMQGA TVG	1397	DVRDWP IQIRGK RK - 1397
mus musculus	1358-	KKPE LGMQGA TVG	1385	DVRDWS IQIRGK RR - 1385
gallus gallus	893-	KRTE LGCPKA TVG	910	DVKDW IQGKGKMK - 910
tetraodon nigroviridans	1350-	KRSQ LKSKS TSVG	1376	DVQDWT IKDWRK K - 1376
trichodesmium erythraeum	439-	N - YQLKDED TTKG	463	EVHQYKFQTKNQ - - - 463

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

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

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

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

Taken together, these findings suggest an independent origin of this mutation, or a very ancient founder. The occurrence of another two different mutations at the same codon (c.4321C>G, p.R1441G in Basque families,^{10,21} and c.4322G>A, p.R1441H in a sporadic PD case¹⁷), is also in keeping with the presence of a mutational hot spot at this position.

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

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

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

***LRRK2* polymorphisms**

We excluded the pathogenic role of 13 novel exonic and intronic variants on the basis of a similar frequency in cases and controls, or of absence of cosegregation with disease (Tables 1–2). On the contrary, the allelic frequency of the intronic variant IVS30 + 12delT was higher in patients than in controls ($P < 0.05$, Fisher Exact test), and two other intronic substitutions (IVS4 –38A>G, IVS5 +33T>C) were detected in patients but not in controls. These variants could not be studied for cosegregation with disease, and their significance in disease causation remains unclear. They might be in LD with other pathogenic variants located in other regions of the *LRRK2* gene, which were not screened in this study. *In silico* analysis (<http://125.itba.mi.cnr.it/~webgene/www.spliceview.html>) showed that none of the intronic variants appear to significantly modify the recognition of the natural splice site. The IVS30 + 12delT variant, as well as other

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

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

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

Novel variants detected in our study are in **bold**. The p.I1371V, p.R1441C, and p.G2019S mutations are highlighted in italic.

Accession number (rs or ss) is given for each known *LRRK2* polymorphism. The nucleotide numbers are according to the *LRRK2* cDNA sequence deposited in Genbank (accession number AY792511).

For each polymorphism, the variant allele is reported after the > symbol, and its allelic frequency in our sample of autosomal dominant PD patients is also given.

*Polymorphisms, which are not present in the database but have been reported previously (Zimprich *et al. Neuron* 2004).

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

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

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

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

When possible, cosegregation of variant with disease was tested. Three intronic substitutions, for which a pathogenic role remains unknown, are highlighted in **bold**.

NA: cosegregation data not available. *Variant in LD with the p.G2019S mutation.

**200 control chromosomes tested.

$P < 0.05$ vs controls, Fisher Exact test.

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

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

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

analysis in a large panel of patients with the p.G2019S mutation²⁷ suggested that IVS13+104 G>A is in strong LD with this mutation.

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

Considerations on the dardarin protein

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

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

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

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

Genotype/phenotype correlations analysis

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

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

Among nine PD patients shown to carry the G2019S mutation, and for whom accurate clinical information is available (data from reference¹²), the mean age at symptoms onset was 54.2 years (range 38–68 years), while the age at last examination in unaffected p.G2019S carriers ($n=6$) was 49.3 years (range 41–58 years). In order to estimate the penetrance of the p.G2019S mutation, we calculated the ratio between the number of affected carriers and the total number of carriers of this mutation at a given age. The values range from 15% at 40 years, to 78% at age 65 years. These findings are in agreement with the reported p.G2019S penetrance in another study,¹⁴ and have important implications for genetic counselling. However, analysis of larger series of families with the p.G2019S mutation is needed in order to define the penetrance of this frequent pathogenic mutation more accurately.

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

Onset age ranged from 33 to 61 years in our family with the p.I1371V mutation, and from 41 to 72 years in the other family with this mutation published previously¹⁸ (though in that family the mutation status was only tested in the proband, with PD onset at age 41 years). In our family, it is possible that the inheritance of additional genetic factors from the father (also affected by PD and not carrying the p.I1371V mutation) contributed in the proband to the onset of PD at a younger age (Figure 2c).

Conclusion

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

Acknowledgements

We thank the patients and family relatives for their contribution, and Tom de Vries-Lentsch for artwork. The DNA samples contributed by the Parkinson Institute – Istituti Clinici di Perfezionamento, Milan, Italy, were from the 'Human genetic bank of patients affected by Parkinson disease and parkinsonisms', supported by Telethon grant n. GTF03009. This study was supported by Grants from the National Parkinson Foundation (Miami, USA), and the Internationaal Parkinson Fonds (The Netherlands) to V Bonifati.

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Appendix

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Supplementary Information accompanies the paper on European Journal of Human Genetics website (<http://www.nature.com/ejhg>)