Non-motor symptoms in Parkinson's disease

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Genotypic and phenotypic characteristics of Dutch patients with early-onset Parkinson's disease

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Movement Disorders in press

Abstract

Background: Early-onset Parkinson's disease (EOPD) has been associated with mutations in the *Parkin, DJ-1, PINK1, LRRK2* and *SNCA* genes.

Objectives: To assess the contribution of these genes in a Dutch EOPD cohort, and the phenotypic characteristics of the mutation carriers.

Methods: A total of 187 unrelated Dutch EOPD patients (age at onset ≤ 50 years) were screened for mutations in all exons of *Parkin, DJ-1* and *PINK1* by direct sequencing and gene dosage analysis. Additionally, analysis of A30P mutation and exon dosage of *SNCA* and sequencing of exons 19, 31, 35, 38, 41 and 48 of *LRRK2* was performed. Phenotypic characteristics were assessed.

Results: Pathogenic variations could explain disease in 4% (7/187) of the patients including six patients carrying homozygous or compound heterozygous mutations in *Parkin* (n=5) or *DJ-1* (n=1) and 1 heterozygous *LRRK2* mutation. We found seven novel mutations. The phenotypic characteristics of mutation carriers varied widely, comparable to the variability seen in sporadic EOPD.

Conclusions: *Parkin* is the most frequently mutated gene in this EOPD cohort, followed by *DJ-1, PINK1* and *LRRK2*. The phenotypic characteristics from mutation carriers were highly variable. The low overall mutation frequency indicates that the extrapolation of mutation frequencies from other populations should be applied with caution.

Introduction

Most cases of Parkinson's disease (PD) present in sporadic form while a minority (~15%) are familial. A few of these families display either typical Mendelian autosomal dominant or recessive inheritance.[1] To date, mutations in five genes have clearly been associated with monogenic forms of PD providing new insights into disease pathogenesis.[1,2] Mode of inheritance is considered autosomal dominant for patients with mutations in *SNCA* (*α-synuclein;*OMIM*163890) and *LRRK2* (*Leucine-rich repeat kinase 2*;OMIM*609007) and autosomal recessive for patients with mutations in *PRKN* (*Parkin*;OMIM*602544), *PINK1* (*PTEN-Induced Putative Kinase 1*;OMIM*608309) and *DJ-1* (*PARK7*;OMIM*602533). Three missense mutations and genomic multiplications of *SNCA* were identified in a few families with early-onset Parkinson's disease (EOPD) yet mutations in this gene are extremely rare.[3] More than 50 variants have been identified in *LRRK2*, including at least 16 pathogenic variants.[3] Mutations in *LRRK2* are common in both early- and late-onset PD occurring in ~5% of familial and 1% of sporadic PD patients[4-6] however, frequencies vary with ethnic background.[3] *Parkin* is the most frequently mutated gene for EOPD with >100 missense mutations and large genomic rearrangements identified so far[3] and the mutation frequency is highly correlated with low age at onset (AAO) and a positive family history. In families with autosomal recessive EOPD, *Parkin* mutations are identified in 28% of patients with an AAO between 46-55 years and in 82% of juvenile patients (AAO ≤ 20).[7] In sporadic EOPD patients, *Parkin* mutations are identified in 3-33% of patients with an AAO ≤ 45-51 years[8-10] and in up to 77% of juvenile patients.[9] *PINK1* mutations are rare in EOPD patients with frequencies ranging from ≤ 1-7%.[3,11] *DJ-1* mutations are also rare as they account for only ≤ 1-2% of EOPD cases.[12-14] The phenotypic characteristics of mutation carriers have been previously described[7-10], although to our knowledge, the whole spectrum of motor and non-motor symptoms has not yet been evaluated. The aim of this study is to evaluate the frequency and nature of mutations in the *Parkin, DJ-1, PINK1, LRRK2* and *SNCA* genes by performing a comprehensive mutation screening in a large,

homogenous EOPD cohort and to evaluate the phenotypic characteristics, including motor and non-motor symptoms, in mutation carriers.

Patients and methods

Study design

This study is part of the "PROfiling PARKinson's disease" (PROPARK) project, a longitudinal cohort study of patients with PD, who are assessed for phenotype, genotype, disability and global outcomes of health, using valid and reliable assessment instruments. Phenotypic data and peripheral blood samples for genotyping were collected between May 2003 and June 2007.

Study participants

Patients fulfilled the United Kingdom PD Society Brain Bank criteria for PD[15] with the exception that positive family history was not regarded as an exclusion criterion. Recruitment procedure has been described elsewhere[16] and was solely based on AAO. The study was approved by the medical ethics committee of the Leiden University Medical Center and all patients gave written informed consent. For the current study, only data from EOPD patients (AAO ≤ 50 years) were used. A total of 375 Dutch controls were screened for the identified novel missense mutations.

Clinical assessments

Patients received a standardized assessment including evaluation of demographic and clinical characteristics, family history and medication. Family history was regarded as positive if the patient recalled first- or second- degree relatives with PD. Measurement instruments for the different clinical domains of PD derived from the SCOPA project (SCales for Outcomes in PArkinson's disease, http://www.scopa-propark.eu/) were previously described.[16] Data obtained for disease severity (Hoehn & Yahr (H&Y)), motor function (SPES/SCOPA-motor), autonomic function (SCOPA-AUT domain scores for urinary, and cardiovascular functioning as well as a sumscore for three gastrointestinal items dealing with constipation), depressive symptoms (Beck

Depression Inventory (BDI)), nighttime sleep and daytime sleepiness (SCOPA-SLEEP NS and DS), cognition (SCOPA-COG), dyskinesias and motor fluctuations (SPES/SCOPA-motor complications), and psychiatric complications (two items assessing hallucinations and illusions from the SCOPA-PC)[17] were used. Motor impairment subtype (tremor or postural instability gait difficulty (PIGD)) and cognitive impairment were determined. The following cut-off values were used to determine if patients were depressed (BDI: 14/15), suffered from excessive nighttime sleep problems (SCOPA-SLEEP NS: 6/7) or from excessive daytime sleepiness (SCOPA-SLEEP DS: 4/5). Patients were considered having autonomic impairment if they had ≥ 50% of the maximum possible score on one of the autonomic domains. Patients who used anti-parkinsonian medication were assessed during "on"-state. For each patient, a total levodopa-equivalent (LDE) was calculated.

Molecular studies

Genomic DNA was extracted from peripheral-blood leukocytes and buccal swabs according to standard procedures from patients and controls, respectively.

Direct Sequencing

All exons and exon-intron boundaries of *Parkin, DJ-1* and *PINK1* and the most frequent mutation-bearing exons of *LRRK2* (19, 31, 35, 38, 41 and 48) were amplified (conditions and primer sequences available upon request). Direct sequencing was performed using BigDye terminator chemistry (Applied Biosystems, Foster City, CA) and sequencing products were processed on an Applied Biosystems 3730 automated DNA sequencer and analyzed using SeqScape software version 2.1 (Applied Biosystems). Nucleotides are numbered according to Genbank accession numbers NM_004562 (*Parkin*), NM_007262 (*DJ-1*), NM_032409 (*PINK1*) and NM_198578 (LRRK2) with A of initiator ATG numbered as +1.

Exon dosage analysis

Patients were screened for exon rearrangements using the multiplex ligation-dependent probe amplification (MLPA) method and SALSA MLPA P051 and P052B Parkinson probe mixes according to the manufacturer's instructions (MRC-Holland b.v.). This probe mix includes probes for all exons of *α-synuclein, Parkin, DJ-1* and *PINK1* genes, except for exons 2 and 4 of *DJ-1*. Additionally, it includes a probe specific for the *SNCA* A30P mutation. Results were analyzed using GeneMarker Sofware (version 1.51, SofGenetics, LLC). Dosage ratio values of ≤ 0.7 and ≥ 1.3 were used as boundaries for deletions and duplications, respectively. One positive control carrying the homozygous deletion involving the first five exons of DJ-1[18] and two negative controls for exon rearrangements were used.

Statistical analysis

If ≥ 25% of the data from a questionnaire or scale was missing for a particular patient, data from this scale was excluded from the statistical analyses. Means, medians, standard deviations, interquartile ranges and percentages were calculated with the Statistical Package for the Social Sciences Software (SPSS version 14.0).

Results

Patient characteristics

In total, 187 EOPD patients (65% male) with a mean AAO of 41.1 ± 6.6 years (range 16 to 50) were assessed for phenotype and genotype. Twenty-two (12%) and 28 (15%) patients had a history of first- and second-degree relatives affected with PD, respectively (Table 1).

Molecular studies

A pathogenic *LRRK2* c.7067C>T;p.T2356I mutation[5] in heterozygous state (Table 2) was found in one patient. None of the patients carried the A30P mutation or exon rearrangements of *SNCA*.

For the recessively inherited phenotypes associated with mutations in *Parkin, PINK1* and *DJ-1* genes, six patients presented with homozygous or compound heterozygous mutations (five in *Parkin*; one in *DJ-1*). Ten carried a single heterozygous mutation (six in *Parkin*; two in *DJ-1*; two in *PINK1*) (Table 2). Additionally, we detected several common non-synonymous changes (not shown) which were reported to be present at a similar frequency in controls, and therefore are unlikely to be pathogenic.

The homozygous mutations in *Parkin* included a deletion of exon 3[9], the pathogenic missense mutation c.125G>C;p.R42P[19] and a novel duplication of exons 10-12. The heterozygous compound mutations were a novel combination of two previously described exon rearrangements, a deletion of exon 4[20] and a duplication of exon 7[9] and the common compound of deletion of exon 3 with the c.823C>T;p.R275W missense mutation.[21] Six patients carried single heterozygous *Parkin* mutations including deletions of exon 3[20], exon 4[20] and exon 3-4[22], c.98G>A;p.R33Q[23], c.1204C>T;p.R402C[10] and a novel mutation in exon 7 (c.848T>C;p.L283P; see Figure 1) not found in 726 control chromosomes. Additionally, we identified one novel synonymous variant in *Parkin* (c.837C>T;p.H279H) (Table 3). In agreement with previous findings[9], gene dosage alterations accounted for the majority of *Parkin* mutations.

Three novel *DJ-1* mutations were identified, a small homozygous deletion of three nucleotides in exon 7 that results in the deletion of a highly conserved proline residue (c.555-557delGCC;p.P158del; see Figure 1), a heterozygous duplication involving the first five exons of *DJ-1* and a heterozygous non-synonymous substitution in exon 7 (c.535G>A;p.A179T; see Figure 1). These mutations were absent in 700 control chromosomes. Additionally, we found few new non-coding variants in *DJ-1* of unknown significance (Table 3).

Two mutations were identified in *PINK1*, a nonsense mutation in exon 7 (c.1474C>T; p.R492X)[24] and a novel missense mutation in exon 3 (c.709A>G;p.M237V; see Figure 1) found in 1 of 750 control chromosomes. Additionally, one patient carried a heterozygous non-coding variant (Table 3).

In total, 4% of the patients carried mutations that could explain PD (7/187).

Table 1. Phenotypic characteristics of patient groups according to number of mutations

Characteristics	Total patient group
No. of patients	187
Age at examination, mean (SD)	52.5 (7.9)
Sex, men/women (% men)	122/65 (65%)
Family with PD, n (%)	
First degree	22 (12%)
Second degree	28 (15%)
Age at onset, mean (SD)	41.1 (6.6)
Age onset 0-20 years, n	3
Age onset >20-30 years, n	10
Age onset >30-40 years, n	54
Age onset >40-50 years, n	120
Disease duration, mean (SD)	11.4 (6.8)
Total LDE, mean (SD)	646.7 (503.3)
H&Y stage, median (IQ range)	2 (2-3)
Motor phenotype, n (%)	
Tremor dominant	78 (42%)
PIGD	94 (50%)
Non determinable	14 (8%)
Cognitive impairment, n (%)	26 (14%)
Depression, n (%)	33 (18%)
Autonomic impairment, n (%)	
Gastrointestinal impairment	32 (17%)
Urinary impairment	34 (18%)
Cardiovascular impairment	3 (2%)
Sleep problems, n (%)	
Nighttime sleep problems	61 (33%)
Daytime sleepiness	75 (40%)
Motor complications, n (%)	
Dyskinesias	50 (27%)
Motor fluctuations	71 (38%)
Psychiatric complications, n (%)	43 (23%)

*: sum of numbers does not equal total group number and sum of percentages does not equal 100 due to missing values

Patients without mutations	Patients with one mutation	Patients with two mutations
170	11	6
52.7 (7.8)	54.5 (8.2)	43.5 (3.6)
111/59 (65%)	7/4 (64%)	4/2 (67%)
16 (9%)	2 (18%)	4 (67%)
27 (16%)	1 (9%)	0 (0%)
41.2 (6.3)	43.1 (7.3)	34.8 (10.4)
2	0	1
9	1	0
49	1	4
110	9	1
11.5 (6.5)	11.4 (9.4)	8.7 (9.4)
671.5 (504.0)	445.3 (502.9)	287.5 (271.9)
2 (2-3)	2 (2-3)	2 (1-3)
*		
68 (40%)	7 (64%)	3 (50%)
89 (52%)	3 (27%)	2 (33%)
12 (7%)	1 (9%)	1 (17%)
23 (14%)	1 (9%)	2 (33%)
30 (18%)	2 (18%)	1 (17%)
29 (17%)	1 (9%)	2 (33%)
29 (17%)	4 (36%)	1 (17%)
3 (2%)	0 (0%)	0 (0%)
56 (33%)	3 (27%)	2 (33%)
74 (44%)	0 (0%)	1 (17%)
48 (28%)	2 (18%)	0 (0%)
66 (39%)	4 (36%)	1 (17%)
41 (24%)	2 (18%)	0 (0%)

PD: Parkinson's disease; LDE: levodopa dosage equivalent; H&Y: Hoehn and Yahr;
IQ: interquartile; PIGD: postural instability gait difficulty

Table 2. Mutations identified in this study

Case No.	Mutation 1	Mutation 2	AAO (years)	Gender	Family History[a]	Family pedigree
Parkin						
1	**R42P**	**R42P**	39,4	M	-	
2	**Ex3Del**	**Ex3Del**	38,1	M	+	brother, uncle and aunt (father side)
3	**Ex3Del[b]**	**R275W[b]**	16,3	F	+	sister
4	**Ex4Del[b]**	**Ex7Duplic[b]**	33,7	F	+	two brothers, half-uncle
5	**Ex10-12Dupl[b]***	**Ex10-12Dupl[b]***	47,5	M	+	father
6	L283P*		48,9	M	-	
7	Ex3Del		42,6	M	-	
8	Ex4Del		27,2	M	-	
9	Ex3-4Del[b]		45,3	F	-	
10	R33Q		48,6	F	+	brother
11	R402C		45,8	M	-	
DJ-1						
12	**P158Del**	**P158Del**	33,8	M	-	
13	A179T*		47,0	M	+	mother
14	Ex1-5Duplic[b]		46,0	F	+	grandmother
PINK1						
15	M237V*		31,1	M	-	
16	R492X		42,8	F	-	
LRRK2						
17	**T2356I**		48,7	M	-	

Mutations considering sufficient to cause the phenotype are indicated in bold.

[a]: with first- or second-degree relative affected (side of the affected family);

[b]: unknown phase; *: novel mutations

AAO: age at onset; M: male; F: female; Del: deletion; Dup: duplication

Clinical characteristics of mutation carriers

Overall, the expression of both motor and non-motor features was variable in mutation carriers (Table 4). The low frequency of mutations precluded statistical comparisons between groups with a different genotype status. Patients were categorized into three groups: (1) with two mutations (homozygote or compound heterozygote), (2) with a single mutation (heterozygote), and (3) without mutations (Table 1). The group 1 had a lower mean AAO, a higher percentage of patients with a positive family history and fewer patients with motor complications (dyskinesias and motor fluctuations) and psychiatric complications compared to the group 3. We also observed an apparent decrease of total LDE and disease duration with an increasing number of mutations (Table 1).

Table 3. Frequency of novel changes identified in this study

Location	nucleotide change	a.a. change	Frequency
Parkin			
	c.837C>T	H279H	0.03
Ex7	c.848T>C	L283P	0.03
Ex10-12Dupl	-	-	0.05
DJ-1			
Intron1A	g.1193G>C	-	0.03
	c.252+46delG	-	0.03
	c.252+47insG	-	0.13
Intron4	c.252+47A>G	-	0.27
Intron5	c.323-159C>T	-	0.03
	c.555-557Del	P158Del	0.05
Ex7	c.535G>A	A179T	0.03
Ex1-5Dupl	-	-	0.03
PINK1			
Intron1	c.387+31A>T	-	0.03
Ex3	c.709A>G	M237V	0.03

Major and minor alleles are shown at each nucleotide variant position. Minor allele frequencies for all variants were calculated.

Table 4. Characteristics of the mutation carriers

Case No.	DD (years)	H&Y	Motor subtype	LDE (mg)	Dyskinesias
Parkin					
1	4.7	1	Tremor	800	-
2	5.8	3	PIGD	300	-
3	27.6	2	ND	225	-
4	7.7	1	PIGD	250	-
5	1.7	2	Tremor	150	-
6	9.1	4	PIGD	1560	-
7	14.4	2	Tremor	480	+
8	30.1	4	PIGD	missing	+
9	2.7	2	Tremor	0	-
10	21.3	2	ND	550	-
11	6.5	2	Tremor	0	-
DJ-1					
12	4.7	3	Tremor	0	-
13	0.3	3	Tremor	0	-
14	3.7	3	Tremor	0	-
PINK1					
15	31.1	2	Tremor	850	-
16	3.0	2	PIGD	337,5	-
LRRK2					
17	18.2	2	Tremor	675	-

DD: disease duration; H&Y: Hoehn and Yahr; PIGD: postural instability gait difficulty;
ND: non-determinable; GI: gastrointestinal dysfunction; UR: urinary dysfunction;
ENSP: excessive nighttime sleep problems; EDS: excessive daytime sleepiness

Motor fluctuations	Depression	Cognitive problems	Autonomic dysfunction	Sleep problems	Psychiatric symptoms
+	-	-	-	-	-
-	+	-	GI and UR	ENSP and EDS	-
-	-	-	GI	-	-
-	-	-	-	ENSP	-
-	-	+	-	-	-
+	+	-	UR	ENSP	-
+	-	-	UR	-	+
+	-	+	-	ENSP	-
-	-	-	-	-	-
-	-	-	GI and UR	-	+
-	-	-	-	-	-
-	-	+	-	-	-
-	+	-	-	ENSP	-
-	-	-	-	-	-
+	-	-	-	-	-
-	-	-	-	-	-
-	-	-	UR	-	-

Figure 1. Conservation of amino acids mutated in novel mutations

Parkin		**283**
H.sapiens	NP_004553	NDRQFVHDPQ**L**GYSLPC----------
P.troglodytes	XP_001153793	NDRQFVHDPQ**L**GYSLPC----------
M.mullata	*XP*_001099588	NDRQFVHDPQ**L**GYSLPCVGTGDIAVLRG
M.musculus	*NP*_057903	NDRQFVHDAQ**L**GYSLPC----------
R.norvegicus	*NP*_064478	NDRQFVHDAQ**L**GYSLPC----------
M.domestica	XP_001381497	NDRQFIHDPV**L**GYSLPC----------
D.rerio	NP_001017635	NERQFTQETL**L**GYSLPC----------
C.elegans	NP_499846	ERFGFVNQPPHGFTIFC----------
D.melanogaster B	NP_730600	GERQFMPHPDFGYTLPC----------

DJ-1		**158**	**179**
H.sapiens	NP_009193	SRG**P**GTSFEFALAIVEALNGKEVA**A**QVK	
P.troglodytes	XP_521268	SRG**P**GTSFEFALAIVEALNGKEVA**A**QVK	
M.mullata	XP_001096631	SRG**P**GTSFEFALAIVEALNGKEVA**A**QVK	
M.musculus	NP_065594	SRG**P**GTSFEFALAIVEALVGKDMANQVK	
R.norvegicus	NP_476484	SRG**P**GTSFEFALAIVEALSGKDMANQVK	
M.domestica	XP_001362447	SRG**P**GTSFEFGLAIIAELMGKSVVDQVK	
D.rerio	NP_001005938	SRG**P**GTSFEFALTIVEELMGAEVA**A**QVK	
C.elegans 1	NP_493696	SRG**P**GTAFEFALKIVELLEGKDKATSLI	
C.elegans 2	NP_504132	SKG**P**GTAFEFALKIVETLEGPEKTNSLL	
D.melanogaster A	NP_610916	SRG**P**GTTFDFALKITEQLVGAEVAKEVA	
D.melanogaster B	NP_651825	SRG**P**GTAYEFALKIAEELAGKEKVQEVA	

PINK1		**237**
H.sapiens	NP_115785	SAGSSSEA-ILNT**M**SQELVPASRVALAG
P.troglodytes	XP_001164912	SAGSSSEA-ILNT**M**SQELVPASRVALAG
M.mullata	XP_001096957	SAGSSSEA-ILNT**M**SQELVPASRVALAG
M.musculus	NP_081156	SAGSSSEA-ILSK**M**SQELVPASRVALAG
R.norvegicus	NP_001100164	SAGSSSEA-ILSK**M**SQEL----------
M.domestica	XP_001377483	SAGSSSEA-IFST**M**SQELVPASRMALSG
D.rerio	XP_001338458	GAGSSSDA-ILRS**M**SMELVPSCPQALRK
C.elegans	NP_495017	EHDRDGDAHLLKS**M**GNELAP--------
D.melanogaster A	NP_572340	DIQSNALS-ILRA**M**YKETVPARQ---RG

Alignment of homologs of Parkin, DJ-1 and PINK1 proteins showing the degree of conservation of the newly identified mutated amino acids which are presented in bold. The position of the amino acids is indicated above the protein sequence.

Discussion

Our results confirm that the A30P missense mutation and multiplication mutations within *SNCA* are a rare cause of EOPD.[25,26] Recent reports indicate that *LRRK2* pathogenic mutations are clustered in only six exons[6], therefore we limited our genetic testing to those exons. In the present patient cohort, only one case was explained by a *LRRK2* mutation. Interestingly, the most common *LRRK2* mutation, G2019S[3], was not detected in our patient cohort, suggesting that this mutation does not play a major role in Dutch EOPD patients.

The combined frequency of *Parkin, DJ-1, PINK1* mutations in EOPD patients was 9% (16/187), including heterozygous carriers. However, since mutations in these genes are associated with autosomal recessive PD, disease could only be attributed to the mutations in homozygote or compound heterozygote cases (3%). *Parkin* mutations were encountered most frequently (6%), while *DJ-1* and *PINK1* mutations were found in 2% and 1% of the cases, respectively. The relatively low overall mutation frequency observed in this cohort in comparison to other studies might be explained by our patient inclusion criteria. Most published estimates are based on EOPD (AAO ≤ 45) families with autosomal recessive inheritance[9], juvenile onset[20], consanguinity[27] and in sporadic cases with AAO ≤ 40.[10] In general, mutations are most frequent within these patient groups, especially in *Parkin*. A genetic screening with similar patient inclusion criteria as in our study, performed in an Italian EOPD cohort reported a similar overall mutation frequency of 8%.[8]

In the present cohort, selection of patients was based solely on AAO, independent of family history resulting in a less biased and restrictive ascertainment. To our knowledge, this study represents the largest cohort screened for mutations in such a comprehensive manner for all EOPD associated genes therefore our findings may provide a reliable estimate of the overall mutation frequency in EOPD in the Netherlands.

Consistent with other studies[9,11,12], most of the mutations found in *Parkin, DJ-1* and *PINK1* genes were heterozygous (62%). However, their pathogenic role remains a matter of debate[28] as phenotypes associated with mutations in these genes are recessively inherited. PET studies of asymptomatic carriers of a single *Parkin* or *PINK1* mutation show a decrease of mean ^{18}F-fluorodopa uptake in the striatum.[28] These results suggest haploinsufficiency or that modest alteration in nigrostriatal function is either tolerated or unrelated to the presence of the detected variant. Moreover, a similar frequency of heterozygous mutations in cases and healthy controls[28], favors the theory that the presence of a single mutation in PD patients is coincidental and unrelated to disease. Due to the gene size, the intronic and regulatory sequences were not fully analyzed; therefore it is possible that some patients have a second undetected mutation. Studies in model organisms and the discovery of PD patients with mutations in both *LRRK2* and *Parkin* or *PINK1* and *DJ-1* suggest that epistasis may play a role in the disease etiology.[3] Evidence of digenic inheritance was not found in our patients. Nevertheless, the identified heterozygous mutations may have epistatic interactions with mutations in other, yet unidentified, genes.

The pathogenicity of R275W variant in *Parkin* is still controversial because this mutation was found to occur as frequently in control subjects (3/192) as in patients (5/313).[21] Notably, this mutation in combination with a truncating mutation is exclusive to patients[21] and is associated with a lower AAO than those with a homozygous deletion.[7,29] Interestingly, the R275W mutation was identified as the most common point mutation in *Parkin* among European patients and it is always associated with a rare allele.[30] Furthermore, this mutation occurs within the RING1 functional domain and leads to large cytoplasmic and nuclear inclusions of the Parkin protein.[31] Overall, the evidence argues against a heterozygous effect of the R275W mutation therefore should not be taken as the cause of disease unless a second mutation is also identified. In light of the above we suggest that the R275W missense mutation in combination with the deletion of exon 3 can explain the disease in patient 3.

Our study revealed a total of seven novel mutations (three in *Parkin*, three in *DJ-1* and one in *PINK1*). One is a large duplication involving the last three exons of *Parkin*

that we classified as homozygous. However, it can not be excluded that it is a heterozygous triplication. Since the parents of this patient are deceased, we have not been able to distinguish between these two possibilities. Another is a compound heterozygous of a deletion of exon 4 with a duplication of exon 7. Exon 7, 10 and 11-12 encode the RING1, IBR and RING2 domains, and therefore the identified genomic rearrangements are likely to produce proteins with alterations in the Parkin E3 ligase activity. We hypothesize that the novel L283P *Parkin* variant, is likely to be pathogenic as it occurs within the functional RING1 domain, the substituted residue is highly conserved, proline is known to be a "helix breaker" and was not detected in controls.

All mutations identified in *DJ-1* are novel. The in-frame homozygous deletion resulting in the loss of the highly conserved proline (P158del) may induce conformational changes rendering *DJ-1* unstable and/or non-functional and is absent in controls. A heterozygous *DJ-1* missense mutation, A179T, that results in the substitution of a hydrophobic alanine by a polar hydrophilic threonine residue, was located within the C-terminal H helix that mediates DJ-1 dimerization.[32] Additionally, we found a heterozygous duplication of the first five exons of *DJ-1*.

The high conservation of residue 237 among *PINK1* homologs and its critical location in the protein kinase domain suggests that the novel M237V variant may affect PINK1 function. The observed frequency is compatible with that of a rare mutation, although we cannot exclude the possibility that it is a rare polymorphism.

The mutation carriers showed a wide phenotypic variability, comparable to the variability seen in sporadic EOPD. The small numbers of mutation carriers precluded statistical comparisons between groups and therefore comparisons with other studies are difficult. Nevertheless, some interesting observations were made. The presence of a single mutation did not decrease AAO as has been suggested previously[9,10,22], however patients with two mutations presented with lower AAO than patients without mutations. Additionally, less patients with two mutations experienced daytime sleepiness, psychiatric and motor complications compared to non-mutation carriers. We could not confirm a higher likelihood of presenting with dyskinesias in *Parkin*

mutation carriers compared to non-mutation carriers as suggested by others.[7,9] Overall, our findings are consistent with previous reports suggesting that patients with *Parkin, DJ-1, PINK1* and *LRRK2* mutations are clinically indistinguishable from age-matched non-mutation carriers with PD.[8]

In conclusion, the relatively low overall mutation frequency indicates that caution has to be taken with the extrapolation of mutation frequencies found in other populations, and suggests that other genes and risk factors for PD remain to be discovered.

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