American Journal of Medical Genetics 129A:44–50 (2004)

parkin Mutation Analysis in Clinic Patients With Early-Onset Parkinson's Disease

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parkin **mutations are the most common identified cause of Parkinson's disease (PD). It has been suggested that patients with young-onset PD be screened for *parkin* mutations as a part of their clinical work-up. The aim of this study was to assess *parkin* mutation frequency in a clinical setting, correlate genotype with phenotype, and evaluate the current justification for clinical *parkin* testing. Patients were selected from a movement disorder clinic based on diagnosis of PD and onset age ≤40 years. *parkin* was genotyped by sequence and dosage analysis for all 12 exons. Key relatives and controls were screened for identified mutations. Mutations were found in 7/39 patients. Two patients were compound heterozygous; five were heterozygous. Mutations included deletions in exons 2, 3, and 8, duplications in exons 2–4, and 9, and P437L substitution. Seventy-eight percent of mutations were deletions/multiplications. A novel substitution (R402W) was found in one patient and in one control. None of the point mutations found in patients were detected in 96 controls. *parkin* phenotypes were consistent with idiopathic PD. In conclusion, *parkin* mutations are common in the clinic setting: 10% of PD patients had early-onset and 18% of them had *parkin* mutations. However, if *parkin* is recessive, only 5% of early-onset cases who had compound mutations could be attributed to this locus. Mutation frequency was 0.12 (95% CI 0.04–0.19). *parkin* cases can present as typical idiopathic PD, distinguishable only by molecular testing. Seventy percent of *parkin* cases were heterozygous. It is unclear whether heterozygous mutations are pathogenic. *parkin*-based diagnosis and counseling require a better understanding of the mode of inheritance, penetrance, and carrier frequencies.** © 2004 Wiley-Liss, Inc.

KEY WORDS: **Parkinson's disease; *parkin*; clinical testing; gene dosage**

INTRODUCTION

Once believed to be non-genetic, Parkinson's disease (PD) has proved to be a heterogeneous disorder with a significant genetic component. In the past 7 years, more than 10 genetic loci have been linked to PD [Gwinn-Hardy, 2002; Kruger et al., 2002], four of which have been identified, and many of the mutations have been characterized. Mutations in α-synuclein [Polymeropoulos et al., 1997] and ubiquitin carboxy-terminal hydrolase L1 [Leroy et al., 1998] cause autosomal dominant PD. Mutations in *parkin* [Kitada et al., 1998] and *DJ-1* [Bonifati et al., 2003] are associated with early-onset recessive PD. *parkin* is the most common single-gene cause of PD known to date.

Localized on chromosome 6 (PARK2 on 6q25-27), *parkin* is a large gene with 12 exons and a 1,395 base-pair (bp) open reading frame [Kitada et al., 1998]. The protein product, Parkin, functions as an ubiquitin E3 ligase in the proteosome degradation pathway [Shimura et al., 2000]. Over 80 *parkin* mutations have been identified, spanning all exons. The mutations include point mutations, as well as deletions and duplications that range in size from a few bp to multiple whole exons [West et al., 2002]. DNA sequence analysis can detect only a subset of mutations. Quantitative gene-dosage analysis is required for detection of heterozygous and compound deletions and multiplications, which comprise 60–80% of all *parkin* mutations [Hedrich et al., 2001; Kann et al., 2002; West et al., 2002; Foroud et al., 2003; Oliveira et al., 2003].

parkin was originally discovered as the genetic locus for autosomal recessive juvenile parkinsonism, a rare disorder that is characterized by onset <20 years, slow disease progression, and marked responsiveness to levodopa. The recent expansion of *parkin* screening to the more common forms of PD has uncovered mutations in patients and families with varied clinical and pathological phenotypes, including families with seemingly autosomal dominant inheritance [Klein et al., 2000; Maruyama et al., 2000; Lucking et al., 2001; Kobayashi et al., 2003], and an autopsy-confirmed *parkin* family with Lewy bodies [Farrer et al., 2001]. The age of onset range has also been expanded, from juvenile and early-onset to beyond age 70 [Lucking et al., 2000; Foroud et al., 2003; Oliveira et al., 2003]. Although *parkin* mutations were presumed recessive,

Grant sponsor: National Institutes of Health; Grant number: RO1 NS36960; Grant sponsor: Veterans' Administration (PADRECC).

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Received 9 July 2003; Accepted 9 December 2003
DOI 10.1002/ajmg.a.30157

recent evidence suggests that heterozygous mutations may also be pathogenic, or may confer increased susceptibility to typical late-onset PD [Farrer et al., 2001; Hedrich et al., 2002; Hilker et al., 2002; West et al., 2002; Oliveira et al., 2003].

The increasing number of reports of *parkin* mutations in patients with common forms of PD suggests a wider involvement of *parkin* in PD than was once believed. These reports raise questions concerning the prevalence of *parkin* mutations, and whether patients with idiopathic PD, at least those with early-onset, should be screened for *parkin* as a part of their clinical work-up [Khan et al., 2003]. Analysis of *parkin* mutations is complex and expensive. Routine clinical genetic testing would not be justified if mutations were rare in the clinical setting. Many studies report that *parkin* mutations are common in early-onset PD. For example, in one study of early-onset PD, 49% of families with recessive inheritance and 18% of isolated cases had *parkin* mutations [Lucking et al., 2000]. Most published estimates, however, are based on special populations selected for genetic studies (e.g., consanguineous families, sib-pairs, and early-onset recessive families in which *parkin* mutations are most prevalent); hence, the mutation rates may have been inflated by selection bias [Kitada et al., 1998; Lucking et al., 2000; Foroud et al., 2003; Oliveira et al., 2003]. A community-based study found *parkin* mutations in 9% of German patients with onset age <50 years [Kann et al., 2002]. The single clinic-based study published to date reports a carrier frequency of 4%, but the authors did not perform gene-dosage studies, and could thus have missed a substantial number of heterozygous and compound deletions and duplications [Chen et al., 2003]. Here, we report the results of a comprehensive *parkin* mutation analysis in a clinical setting. We studied every clinic patient who had the diagnosis of idiopathic PD with early age at onset, and who agreed to participate in the study, regardless of family history. We sequenced all 12 exons and performed quantitative gene-dosage analysis for all exons, to detect homozygous, heterozygous, and compound deletions, multiplications, and point mutations. This study represents a first step in assessing the justification for *parkin* screening in the clinical settings.

MATERIALS AND METHODS

Patients

Study subjects were patients who were seen by neurologists at the movement disorder clinic at Oregon Health & Science University (OHSU), and had received a clinical diagnosis of PD according to the British Parkinson's Disease Brain Bank criteria except that family history was not an exclusion criteria [Hughes et al., 1992]. The majority of patients are living, thus lack pathological confirmation.

Subjects were initially identified and invited to join the study solely on the basis of having the diagnosis of PD, regardless of age at onset or family history. Informed consent was obtained with Institutional Review Board (IRB) approval from OHSU. Each subject was asked to provide a family history and a blood sample for DNA preparation. Family history was obtained using a self-administered questionnaire, and if positive for PD or other neurological disorders, they were verified by neurological examination of the family members or by review of existing medical records. Key family members were identified and enrolled with IRB-approved informed consent. Ages at onset were recorded upon entering the study and were cross-checked against the earlier medical records. Early-onset was defined as $\leq$40 years because *parkin* mutations are predominantly found in this group [Lucking et al., 2000].

Four hundred forty two patients with diagnosis of PD were enrolled in the study and donated DNA (431 Caucasian, 1 Asian, 1 African-American, 3 Hispanic, 5 native American, 1 mixed ethnicity). For 43 subjects, age at onset was unambiguously established at or before age 40. Four of 43 subjects had insufficient DNA hence were excluded. *parkin* analysis included 39 subjects (22 male, 17 female), representing all consenting clinic patients with diagnosis of PD, early-onset, and sufficient DNA. We did not investigate patients with later onsets because at the time the literature suggested that *parkin* mutations were not involved in late-onset disease [Oliveri et al., 2001]. All 39 subjects analyzed were Caucasian, residing in the Pacific Northwest. Their ages at onset ranged from 7 to 40 years (32.6 ± 8.1 years). Their ages at enrollment/blood draw ranged from 36 to 81 years (52.5 ± 9.9 years).

Controls

Ninety-six unaffected Caucasian volunteers were used as control subjects. Like the patients, controls were Caucasians from the Pacific Northwest. They were ascertained and enrolled under protocols approved by the IRB of the University of Washington. Their ages at enrollment and blood draw ranged from 18 to 72 years (37.9 ± 12.1 years).

parkin DNA Sequence Analysis

To identify point mutations, we sequenced both DNA strands of all 12 exons of the *parkin* gene. Genomic DNA from blood was used. Primer pairs for amplification and sequencing have been described previously [Kitada et al., 1998]. Exons along with 50–100 bp of flanking intronic sequences were PCR-amplified, agarose gel-purified (Gene-clean III, Bio101), and directly sequenced by dye-terminator cycle sequencing (ABI, Big-Dye) using an ABI377 sequencer. Nucleotide changes that had been found in patients were screened for in controls, by sequencing the entire exon, or by a restriction assay for the specific change.

Gene-Dosage Assays

To identify exon deletions and duplications, we analyzed gene dosage using real-time fluorescence-based PCR (ABI 7700 Sequence Detector). Amplification of subject genomic DNA was performed using fluorescently labeled probes (5′ FAM or VIC, 3′ TAMRA) and Taqman Universal PCR Mix (ABI) [Maruyama et al., 2000; Tsuang et al., 2002]. *parkin* exon amplifications were multiplexed under standard conditions with an 84 bp fragment of a single-copy human β-actin gene (Genbank accession number XM_004814) as an internal control. A standard curve was generated for each *parkin* exon and for β-actin using 0, 5, 15, 55, and 220 ng of control human genomic DNA. The number of PCR cycles required before the ABI 7700 detects each *parkin* exon product (CT value) was plotted against the corresponding exon standard curve, thus calculating the relative *parkin* copy number. The copy number for each exon was normalized to the single-copy actin gene within each multiplexed reaction and to a normal control reference individual, allowing an estimate of the number of copies of *parkin*. Optimal threshold levels for each primer set were maintained between plate analyses. All controls and samples were analyzed in triplicate.

R402W Restriction Digest Assay

Because we found a novel substitution, R402W, in one patient, we developed a restriction digest assay to detect it. A *BsiWI* site encompassing the mutated nucleotide was artificially introduced, by changing the underlined nucleotides in the reverse detection primer: R402W R 5′-GGTTTCTTT-GGAGGCTGCTTCCGTAC-3′. PCR amplification with the 11F/R402W R primer set amplifies a 156 bp product, where the normal A allele digests with *BsiWI*, yielding two fragments of 22 and 134 nt, and the mutant T allele does not digest. PCR

reactions were performed in 25 μl reaction volumes, and restriction digests were carried out directly in the PCR buffer with the addition of 10× restriction enzyme buffer (2.9 μl), and 10 U of enzyme. Fragments were resolved by gel electrophoresis using 4% Nusieve gels with ethidium bromide.

Haplotype Analysis

One patient and her sister (kindred C, Fig. 1) had a 40 bp deletion in exon 3, as did two previously published families, Ph and Pw [Farrer et al., 2001]. To determine if the E3 40 bp deletion haplotype that we found was the same as the E3 40 bp deletion haplotype in the published families, we genotyped six members of kindred C, including three affected and three unaffected subjects, for nine markers within and spanning the *parkin* locus on 6q25.2-27. Genotyping was performed as previously published for the following markers: D6S437, D6S1581, D6S305, D6S980, D6S1599, D6S1277, D6S264, and D6S1027 [Farrer et al., 2001].

RESULTS

Seven of the 39 patients analyzed had *parkin* mutations (Table I). DNA sequence analysis identified two known mutations in three patients (P437L and 40 bp del 438–477), a novel variant (R402W), and several polymorphisms (Table II). P437L and del 438–477 were not found in the 96 controls and have been previously reported as pathogenic changes [Farrer et al., 2001; Hedrich et al., 2002]. Gene-dosage analysis identified five patients with deletions or duplications. These included deletions in exons 2, 3, and 8, and duplications in exons 2–4, and 9 (Table I). Deletions and duplications comprised 78% of the mutations. *parkin* mutation frequency in early-onset PD was 0.12 (95% confidence interval: 0.04–0.19).

We found eight previously reported polymorphisms, and one novel rare variant (Table II). The novel R402W substitution was found in one patient and in one control. Both individuals were heterozygous. It is not clear if this change is associated with disease, or is simply a rare variant. We could

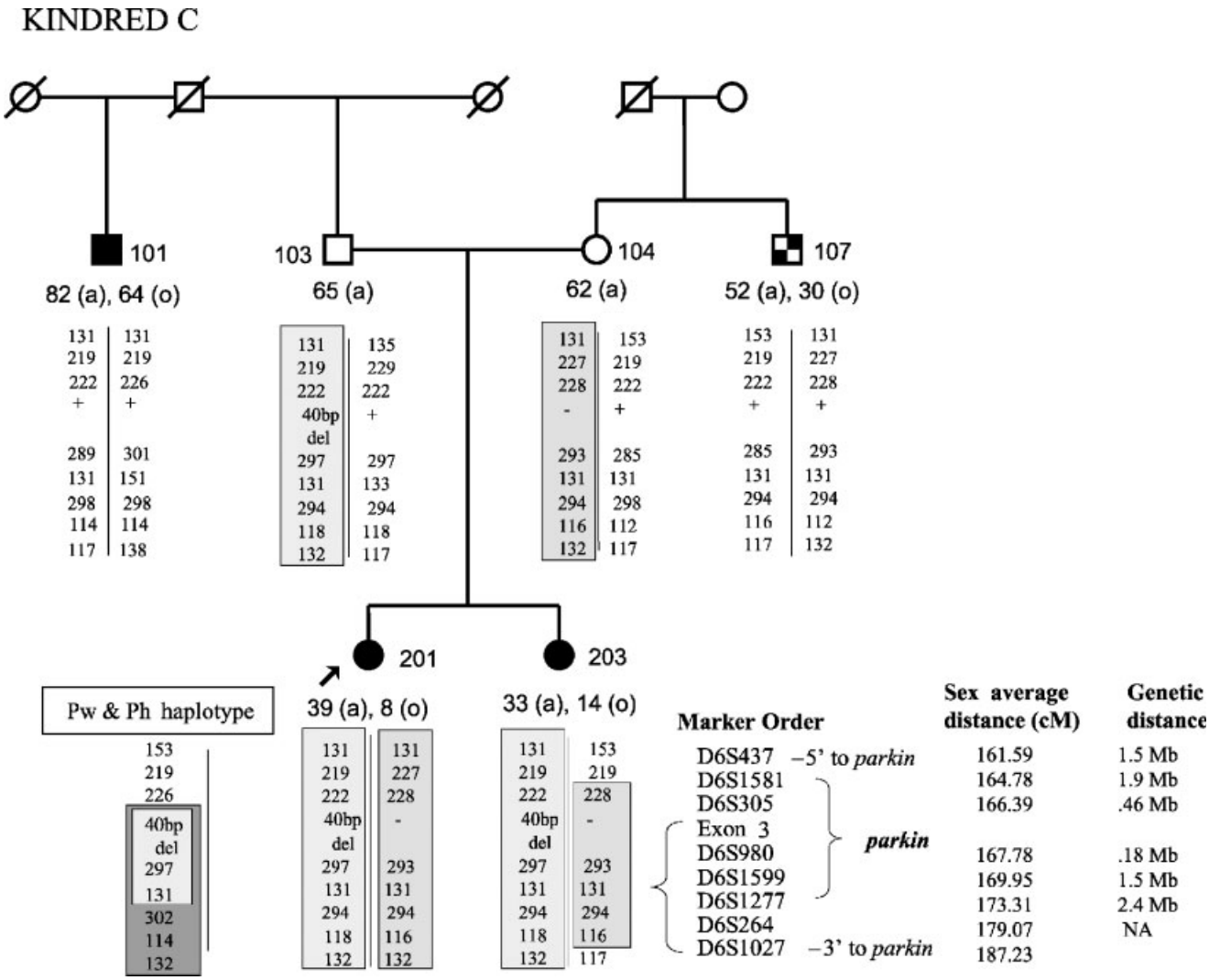

Fig. 1. Haplotype analysis of kindred C. To determine if the *parkin* E3 40 bp deletion in kindred C is within the same haplotype as the E3 deletions in the Ph and Pw families [Farrer et al., 2001], we used identical chromosomal markers encompassing and within *parkin* to genotype six family members, including the two affected siblings with compound *parkin* mutations, the unaffected parents, a paternal uncle with parkinsonism and a maternal uncle with tremor. Marker locations are from the sex averaged Marshfield map, http://research.marshfieldclinic.org/Map_Markers/maps/IndexMapFrames.html. Genetic distances, as listed, between the markers were derived from a human chromosome 6 genomic contig containing *parkin* (Genbank accession no. NT_007422). Haplotypes were manually reconstructed. The Ph and Pw shared haplotype is shown in the figure. Solid symbol, PD; hatched symbol, tremor; a, current age; o, onset age.

TABLE I. *parkin* Mutations Identified in 7 of 39 Patients With Early-Onset PD

Subject ID	Age of onset	Sex	Family history	Mutation 1	Mutation 2
C 201	8	F	Positive	40 bp del E3	del E3
D 201	37	M	Positive	del E2	del E3
E 302	30–32	M	Positive	dup E2-4[a]	[a]
F 201	25	F	Negative	del E8	None[b]
G 201	37	F	Negative	dup E9	None[b]
H 203	14	F	Negative	P437L E12	None[b]
I 201	37	F	Negative	P437L E12	None[b]

[a]Unable to set phase.
[b]Second mutation was not detected by sequence analysis and dosage assays for all exons.

not determine if it segregated with disease because the patient had a negative family history. The control subject with this substitution had no apparent neurological symptoms and reported no family history of neurological disease.

Of the 39 early-onset cases genotyped, 16 had a positive family history, 22 were sporadic, 1 was unknown. The 7 among the 39 who had *parkin* mutations included four of 22 sporadic cases and three of 16 familial cases. Two patients had compound heterozygous mutations; five were heterozygous. None of the patients had homozygous mutations. None of the subjects with *parkin* mutations have had autopsy documentation. We studied the key relatives of *parkin* carriers to set phase, to study segregation of the mutations in the families, and to correlate genotype with phenotype, as described below for each case.

Subject C 201 (Kindred C)

Onset age 8, compound heterozygous (40 bp del E3 and del E3), positive family history (Fig. 1). Subject C 201 had a 40 bp deletion in E3 on one chromosome and a deletion of the entire E3 on the other chromosome. The proband (C 201) and her sister (C 203) were diagnosed with early-onset PD, and their paternal uncle (C 101) had the diagnosis of late-onset PD (Fig. 1). Ages at onset were 8, 14, and 64 years, respectively. We performed full *parkin* sequence and dosage analysis on the affected sister (C 203) and screened the paternal uncle (C 101), the unaffected parents (C 103 and C 104), and a maternal uncle with isolated tremor (C 107). The affected sister (C 203) had the same compound heterozygous genotype as did the proband. The parents were heterozygous; one had the 40 bp deletion, the other had the E3 deletion. The paternal uncle with late-onset PD had no *parkin* mutations. The maternal uncle with tremor did not have any mutations, either.

The 40 bp deletion (del 438–477) has been previously reported to lie within a conserved haplotype in two other families (Ph and Pw) [Farrer et al., 2001]. To determine if kindred C in our study had the same founder haplotype, we genotyped six members of the kindred with the same nine markers used by Farrer et al. [2001] to establish haplotype. The 40 bp deletion haplotype in kindred C, as depicted in Figure 1, is not the same as the haplotype of the Ph and Pw kindreds, except for two markers at 3′ end of the deletion (D6S1599 and D6S980). Allele 131, which is shared by the three families, is the commonest allele of D6S1599 with the frequency of 0.5. Allele frequencies for D6S980 are not available. All three families are Caucasian of European decent: Pw is German, Ph is Irish, kindred C is Swedish and English. Although the extended haplotypes are not identical, a common old ancestry cannot be excluded.

The proband (C 201) developed dystonia in the right foot at age 8, which became generalized over the following 2 years. Hyperreflexia, rigidity, and tremor were noted. She was treated with pyridoxine and trihexyphenidyl (up to 100 mg/day) with some improvement in symptoms. The dystonia was slowly replaced by PD (fine postural tremor, rigidity, and marked bradykinesia). At age 24 she was started on dopaminergic agents, first a dopamine agonist and then carbidopa/levodopa, which resulted in a dramatic improvement. From age 28 to 39 (present age) she has had no dystonia and only parkinsonism ("off" Hoehn and Yahr stage 4) complicated by marked levodopa-induced motor fluctuations and dyskinesia. She takes 1,050 mg of levodopa per day. The proband's younger sister (C 203, younger by 5 years) developed dystonia which manifested as ill-defined difficulty with running, carrying one shoulder higher and a mild tremor in her hands at age 14. At age 20, she was recognized to have parkinsonism and dystonia, and levodopa treatment was initiated. When first seen in our clinic at age 24 she had spasmodic dysphonia, writer's cramp, mild rigidity, and asymmetric bradykinesia. At age 33 (present age) she has spasmodic dysphonia and parkinsonism ("off" Hoehn and Yahr stage 3) that is treated with 1,800 mg of levodopa per day. Treatment is complicated by levodopa-induced motor fluctuations and dyskinesia. Neither sister has been able to tolerate dopamine agonists because they induce hypomanic behavior.

TABLE II. *parkin* Polymorphisms and Their Frequencies in Patients and Controls

Polymorphism	Frequency of rare allele (no. of chromosomes tested)		
	Patients (this study)	Controls (this study)	Controls [Abbas et al., 1999]
Coding sequence polymorphism			
S167N	0.01 (78)	0.02 (158)	0.01 (166)
V380L	0.17 (78)	0.11 (154)	0.16 (90)
D394N	0.01 (78)	0.07 (152)	0.07 (180)
R402W	0.01 (78)	0.005 (192)	—
Intronic polymorphism			
IVS2 + 25t-c	0.26 (78)	0.27 (176)	0.19 (90)
IVS3-20c-t	0.12 (76)	0.07 (190)	0.10 (90)
IVS7-35a-g	0.38 (78)	0.47 (184)	0.27 (72)
IVS8 + 48c-t	0.13 (60)	0.19 (184)	—
IVS7-68g-c	0.13 (78)	0.23 (182)	—

Subject D 201

Onset age 37, compound heterozygous (del E2 and del E3), positive family history. To determine if the deletions of E2 and E3 were contiguous on the same chromosome, or if they were compound heterozygous deletions, we analyzed genomic DNA from the subject's parents. Both parents carried heterozygous deletions, one with an E2 deletion, and the other with an E3 deletion; therefore, D 201 carries compound heterozygous *parkin* mutations.

The proband (D 201) had onset of symptoms at age 37 and was diagnosed with PD at age 42. He had one affected uncle who had an age at onset of 41 and was diagnosed with PD at age 43. The uncle was deceased and no DNA was available. D 201 has Hoehn and Yahr stage 2 parkinsonism with minimal tremor and asymmetrical bradykinesia and rigidity. He was started on carbidopa/levodopa at age 44; has been maintained on low dose (75/300 mg/day) for 9 years with almost complete abolition of his parkinsonism and no motor fluctuations or dyskinesia.

Subject E 302

Onset age 30–32, dup E2-4, positive family history. This subject had the onset of right arm rest and postural tremor at about age 30–32 and was diagnosed as having PD at age 34 when rigidity and bradykinesia were evident in the right arm. He was started on levodopa at age 38 and when last seen at age 52, he continued to respond to the drug (Hoehn and Yahr stage 3) and experienced motor fluctuations and dyskinesia. He suffered with depression. Proband has two affected sibs (E 304 and E 305). E 304 had the onset of a rest and postural tremor at age 27. Levodopa was initiated at age 34 and he now has dyskinesia and motor fluctuations. Psychiatric evaluation for depression and anxiety suggested that he had a schizoid personality. E 305 had onset of prominent rest and postural tremor at age 24. She was started on levodopa which reduced the tremor but at age 39 she had fluctuations in response and dyskinesia. Depression and anxiety with panic attacks complicated her course.

Subject F 201

Onset age about 25, heterozygous, negative family history. The subject had a heterozygous deletion in exon 8. Although family history is negative for PD, F 201 has a half-sister with isolated tremor. Suspecting that tremor may be a variable expression of the same E8 deletion, we genotyped the half-sister. She did not have the deletion. F 201 had the onset of PD at around age 25 with an asymmetrical rest tremor followed by rigidity and bradykinesia in the tremulous arm. Treatment with levodopa improved her symptoms but at age 52, when last seen, she experienced marked motor fluctuations with an "off" Hoehn and Yahr severity of 4 and prominent dyskinesia.

Subject G 201

Onset age 37, heterozygous, negative family history. This subject has a heterozygous duplication of exon 9. She had the onset of PD at age 37 with right hand rest and postural tremor. Over the following 15 years, the tremor has become severe and bilateral, although asymmetric, as well as more generalized bradykinesia and a shuffling gait. She started levodopa at age 48; within 2 years, motor fluctuations and dyskinesias, mainly affecting the right extremities, have developed as well. At age 52 she has prominent rest and postural tremor when off and is Hoehn and Yahr stage 3.

Subject H 203

Onset age 14, heterozygous single base substitution in exon 12 (P437L), negative family history. This patient has an asymmetrical, slowly progressive, tremor predominant parkinsonism. At age 80, she was Hoehn and Yahr stage 3 with asymmetrical rest tremor, rigidity, and bradykinesia despite using no antiparkinson medications because she did not like the subjective effects she experienced with levodopa.

Subject I 201

Onset age 36, heterozygous single base substitution in exon 12 (P437L), negative family history. She had the onset of PD at age 36 with bradykinesia, rigidity and a postural tremor. She was started on carbidopa/levodopa at age 38 and has had a definite response but complicated by marked motor fluctuations, dyskinesia and a peculiar "on" hypotonia and ataxic state without dyskinesia. Currently at age 57, she has had a unilateral pallidotomy, a subsequent bilateral subthalamic stimulation, and continues to take 1,200 mg of levodopa per day to control her parkinsonism which is Hoehn and Yahr stage 4 when "off".

DISCUSSION

In a movement disorder clinic, seven of 39 patients (18%) with early-onset PD had *parkin* mutations. Early-onset cases comprised 10% of the clinic's patient population. Several of the *parkin* cases presented as typical idiopathic PD, distinguishable only by mutation analysis. Two-thirds of the *parkin*-positive patients were missed by DNA sequencing alone and required detection by quantitative gene-dosage assays. Among the *parkin*-positive cases, 71% were heterozygous, and 29% were compound heterozygous. The carrier frequency (percentage of early-onset subjects with one or two mutations) was 18%. Assuming a strictly recessive inheritance, only 5% of patients with early-onset PD had a pathogenic *parkin* genotype; the remaining 13% were heterozygous. Whether these heterozygous *parkin* mutations were the cause of the disease in these patients is unknown.

Recent studies have proposed that heterozygous mutations may also be pathogenic, or may lead to later-onset disease [Farrer et al., 2001; Hedrich et al., 2002; Hilker et al., 2002; West et al., 2002; Oliveira et al., 2003]. The majority of patients who have *parkin* mutations are heterozygous. In prior studies, 60–75% of early-onset *parkin*-positive patients, and 82% of late-onset *parkin*-positive patients were heterozygous [Hedrich et al., 2002; Kann et al., 2002; West et al., 2002; Foroud et al., 2003; Oliveira et al., 2003]. In our study, 71% of *parkin*-positive patients were heterozygous. The mutations that we found in heterozygous patients have been reported before in patients with single and compound mutations and are considered pathogenic when occurring in combination with another pathogenic mutation [Lucking et al., 2000; Hedrich et al., 2001; Kann et al., 2002; West et al., 2002]. Whether these "pathogenic" mutations can cause disease in the heterozygous state is unknown. Due to the enormity of the gene and its intervening sequences, it has not been feasible to fully analyze the intronic and regulatory sequences. Therefore, it remains possible that the seemingly heterozygous subjects have a second undetected mutation embedded in an intron or other regulatory regions outside the coding sequence. It is also possible that the presence of a single mutation in some patients is coincidental, and unrelated to disease. Control subjects were sequenced, but they were not tested for mutations involving gene dosage. None of the point mutations found in our patients were found in normal controls, in either the present study or other published studies [Broussolle et al., 2000; Farrer et al., 2001; Hedrich et al., 2002; Kann et al., 2002]. However, the majority of mutations are heterozygous deletions and duplications detectable only by dosage analysis, for which there are no data for normal controls in the literature. Thus, the carrier frequency in normal control population is largely unknown and it may not significantly exceed the frequency of heterozygous patients. In short, the heterozygous genotypes could be the cause of the disease, could be unrelated to disease, or could only appear to be heterozygous because we were unable to detect the second mutation.

Prior estimates of *parkin* carrier frequency for early-onset PD have ranged widely from 4 to 49%, varying primarily by patient selection criteria and mutation detection methods used. If we consider the differences in subject selection and inclusion criteria, and their expected consequences on results, our findings are in line with prior reports. Carrier frequency in our specialty clinic (18%) was higher than a community-based study which included patients with onset ages up to 50 years (9%) [Kann et al., 2002], similar to rates found for other isolated or familial cases with early-onset PD (16–18%) [Lucking et al., 2000; Hedrich et al., 2002; Oliveira et al., 2003], and lower than rates in families with early-onset and recessive inheritance (49%) [Lucking et al., 2000]. We found significantly more *parkin* carriers among early-onset patients than did another

recent clinic-based study, which found a carrier frequency of only 4% in 27 patients screened [Chen et al., 2003]. The study did not analyze gene dosage and therefore may have missed some of the mutations. Our results would be comparable if we had calculated the carrier frequency based only on the mutations that were detectable by sequence analysis.

The majority of *parkin* mutations are found in patients with early-onset PD, and yet, the majority (82% in our study) of early-onset PD do not have *parkin* mutations. This underscores the heterogeneity within idiopathic PD, and the need for further elucidation of additional causes. To further appreciate the complexity of idiopathic PD, one should keep in mind that early-onset PD is only a small subtype in the overall picture. *parkin* mutations have been found in the more typical late-onset disease, but their frequency appears to be much lower [Foroud et al., 2003; Oliveira et al., 2003]. The causes of common, late-onset PD are still unknown.

The clinical phenotype of *parkin*-positive patients was remarkably similar to that of patients without *parkin* mutations. Although certain features may be more common in *parkin* families, *parkin* cases cannot be distinguished solely on the basis of clinical features and require genetic testing [Bonifati et al., 2001; Foroud et al., 2003; Khan et al., 2003; Periquet et al., 2003]. Both the compound heterozygotes and the heterozygotes had clinical features that are characteristic of young-onset PD. Response to levodopa in all subjects was generally excellent, although the complications of long-term levodopa therapy were common and severe [Quinn et al., 1987; Schrag et al., 1998]. Tremor often had postural and rest components and was frequently prominent. Levodopa responsiveness and levodopa-induced complications were also common in the study of Lucking et al. [2000]. Two kindreds in particular stood out among our clinic-based population. Kindred C was marked by childhood and juvenile onset cases with prominent dystonia, and kindred E had a large familial component and very prominent tremor. Nevertheless, when these subjects were seen in our clinic, the clinical picture was consistent with idiopathic PD.

It has been proposed that *parkin* should be a part of diagnostic work-up for young-onset parkinsonism [Khan et al., 2003]. The need for complex quantitative dosage assays [Hedrich et al., 2001], the large size of the *parkin* gene [Kitada et al., 1998], the wide variety of the mutations observed to date [Abbas et al., 1999; West et al., 2002], and the frequent de novo rearrangements [Periquet et al., 2001] all make *parkin* analysis technically difficult and expensive. Clinical interpretation of results, particularly for heterozygous genotypes, is another major challenge. *parkin*-based diagnosis may be informative for a small subset of cases that have homozygous or compound mutations. However, the majority of *parkin*-positive patients are heterozygous, and the pathogenecity of heterozygous mutations has not been established. No data are available on sensitivity and specificity of these mutations. Consider a patient with two *parkin* mutations who seeks counseling for family planning: just a few years ago, when *parkin* was presumed to be recessive, he would have been advised that his children are not at risk, whereas in fact, depending on the penetrance of heterozygous mutations, each child may be at substantial risk. Wide application of clinical *parkin* testing would be premature, until the mode of inheritance, penetrance and population carrier frequencies are established. Determining the diagnostic and predictive value of *parkin* may be a daunting task, if the nature and degree of disease association vary by the type, dosage, and combination of mutations.

ACKNOWLEDGMENTS

The authors thank the patients, families, and volunteers who participated in the study.

REFERENCES

Abbas N, Lucking C, Ricard S, Durr A, Bonifati V, De Michele G, Bouley S, Vaughan J, Gasser T, Marconi R, Broussolle E, Brefel-Courbon C, Harhangi B, Oostra B, Fabrizio E, Bohme G, Pradier L, Wood N, Filla A, Meco G, Denefle P, Agid Y, Brice A. 1999. A wide variety of mutations in the parkin gene are responsible for autosomal recessive parkinsonism in Europe. Hum Mol Genet 8:567–574.

Bonifati V, De Michele G, Lucking CB, Durr A, Fabrizio E, Ambrosio G, Vanacore N, De Mari M, Marconi R, Capus L, Breteler MM, Gasser T, Oostra B, Wood N, Agid Y, Filla A, Meco G, Brice A. 2001. The parkin gene and its phenotype. Italian PD Genetics Study Group, French PD Genetics Study Group and the European Consortium on Genetic Susceptibility in Parkinson's Disease. Neurol Sci 22(1):51–52.

Bonifati V, Rizzu P, van Baren MJ, Schaap O, Breedveld GJ, Krieger E, Dekker MC, Squitieri F, Ibanez P, Joosse M, van Dongen JW, Vanacore N, van Swieten JC, Brice A, Meco G, van Duijn CM, Oostra BA, Heutink P. 2003. Mutations in the *DJ-1* gene associated with autosomal recessive early-onset parkinsonism. Science 299(5604):256–259.

Broussolle E, Lucking C, Ginovart N, Pollak P, Remy P, Durr A. 2000. [18 F]-dopa PET study in patients with juvenile-onset PD and parkin gene mutations. Neurology 55:877–879.

Chen R, Gosavi NS, Langston JW, Chan P. 2003. Parkin mutations are rare in patients with young-onset parkinsonism in a US population. Parkinsonism Relat Disord 9(5):309–312.

Farrer M, Chan P, Chen R, Tan L, Lincoln S, Hernandez D, Forno L, Gwinn-Hardy K, Petrucelli L, Hussey J, Singleton A, Tanner C, Hardy J, Langston JW. 2001. Lewy bodies and parkinsonism in families with parkin mutations. Ann Neurol 50(3):293–300.

Foroud T, Uniacke SK, Liu L, Pankratz N, Rudolph A, Halter C, Shults C, Marder K, Conneally PM, Nichols WC. 2003. Heterozygosity for a mutation in the parkin gene leads to later onset Parkinson disease. Neurology 60(5):796–801.

Gwinn-Hardy K. 2002. Genetics of parkinsonism. Mov Disord 17(4):645–656.

Hedrich K, Kann M, Lanthaler AJ, Dalski A, Eskelson C, Landt O, Schwinger E, Vieregge P, Lang AE, Breakefield XO, Ozelius LJ, Pramstaller PP, Klein C. 2001. The importance of gene dosage studies: Mutational analysis of the parkin gene in early-onset parkinsonism. Hum Mol Genet 10(16):1649–1656.

Hedrich K, Marder K, Harris J, Kann M, Lynch T, Meija-Santana H, Pramstaller PP, Schwinger E, Bressman SB, Fahn S, Klein C. 2002. Evaluation of 50 probands with early-onset Parkinson's disease for Parkin mutations. Neurology 58(8):1239–1246.

Hilker R, Klein C, Hedrich K, Ozelius LJ, Vieregge P, Herholz K, Pramstaller PP, Heiss WD. 2002. The striatal dopaminergic deficit is dependent on the number of mutant alleles in a family with mutations in the parkin gene: Evidence for enzymatic parkin function in humans. Neurosci Lett 323(1):50–54.

Hughes A, Daniel S, Kilford L, Lees A. 1992. Accuracy of clinical diagnosis of idiopathic Parkinson's disease: A clinico-pathological study of 100 cases. J Neurol Neurosurg Psychiatry 55:181–184.

Kann M, Jacobs H, Mohrmann K, Schumacher K, Hedrich K, Garrels J, Wiegers K, Schwinger E, Pramstaller PP, Breakefield XO, Ozelius LJ, Vieregge P, Klein C. 2002. Role of parkin mutations in 111 community-based patients with early-onset parkinsonism. Ann Neurol 51(5):621–625.

Khan NL, Graham E, Critchley P, Schrag AE, Wood NW, Lees AJ, Bhatia KP, Quinn N. 2003. Parkin disease: A phenotypic study of a large case series. Brain 126(Pt 6):1279–1292.

Kitada T, Asakawa S, Hattori N, Matsumine H, Yamamura Y, Minoshima S, Yokochi M, Mizuno Y, shimizu N. 1998. Mutations in the parkin gene cause autosomal recessive juvenile parkinsonism. Nature 392:605–608.

Klein C, Pramstaller P, Kis B, Page C, Kann M, Leung J, Woodward H, Castellan C, Scherer M, Vieregge P, Breakefield X, Kramer P, Ozelius L. 2000. Parkin deletions in a family with adult-onset, tremor-dominant parkinsonism: Expanding the phenotype. Ann Neurol 48:65–71.

Kobayashi T, Matsumine H, Zhang J, Imamichi Y, Mizuno Y, Hattori N. 2003. Pseudo-autosomal dominant inheritance of PARK2: Two families with parkin gene mutations. J Neurol Sci 207(1–2):11–17.

Kruger R, Eberhardt O, Riess O, Schulz JB. 2002. Parkinson's disease: One biochemical pathway to fit all genes? Trends Mol Med 8(5):236–240.

Leroy E, Auburger G, Leube B, Ulm G, Mezey E, Harta G, Brownstein M, Jonnalagada S, Chernova T, Dehejia A, Lavedan C, Gasser T, Steinbach P, Winkinson K, Polymoropoulos M. 1998. The ubiquitin pathway in Parkinson's disease. Nature 395:451–452.

Lucking CB, Durr A, Bonifati V, Vaughan J, De Michele G, Gasser T, Harhangi BS, Meco G, Denefle P, Wood NW, Agid Y, Brice A. 2000. Association between early-onset Parkinson's disease and mutations in the parkin gene. French Parkinson's Disease Genetics Study Group. N Engl J Med 342(21):1560–1567.

Lucking CB, Bonifati V, Periquet M, Vanacore N, Brice A, Meco G. 2001. Pseudo-dominant inheritance and exon 2 triplication in a family with parkin gene mutations. Neurology 57(5):924–927.

Maruyama M, Ikeuchi T, Saito M, Ishikawa A, Yuasa T, Tanaka H, Hayashi S, Wakabayashi K, Takahashi H, Tsuji S. 2000. Novel mutations, pseudo-dominant inheritance, and possible familial affects in patients with autosomal recessive juvenile parkinsonism. Ann Neurol 48:245–250.

Oliveira SA, Scott WK, Martin ER, Nance MA, Watts RL, Hubble JP, Koller WC, Pahwa R, Stern MB, Hiner BC, Ondo WG, Allen FH Jr, Scott BL, Goetz CG, Small GW, Mastaglia F, Stajich JM, Zhang F, Booze MW, Winn MP, Middleton LT, Haines JL, Pericak-Vance MA, Vance JM. 2003. Parkin mutations and susceptibility alleles in late-onset Parkinson's disease. Ann Neurol 53(5):624–629.

Oliveri RL, Zappia M, Annesi G, Bosco D, Annesi F, Spadafora P, Pasqua AA, Tomaino C, Nicoletti G, Pirritano D, Labate A, Gambardella A, Logroscino G, Manobianca G, Epifanio A, Morgante L, Savettieri G, Quattrone A. 2001. The parkin gene is not involved in late-onset Parkinson's disease. Neurology 57(2):359–362.

Periquet M, Lucking C, Vaughan J, Bonifati V, Durr A, De Michele G, Horstink M, Brefel-Couborn C, Denefle P, Meco G, Gasser T, Breteler M, Wood N, Agid Y, Brice A. 2001. Origin of the mutations in the parkin gene in Europe: Exon rearrangements are independent recurrent events, whereas point mutations may result from founder effects. Am J Hum Genet 68:617–626.

Periquet M, Latouche M, Lohmann E, Rawal N, De Michele G, Ricard S, Teive H, Fraix V, Vidailhet M, Nicholl D, Barone P, Wood NW, Raskin S, Deleuze JF, Agid Y, Durr A, Brice A. 2003. Parkin mutations are frequent in patients with isolated early-onset parkinsonism. Brain 126(Pt 6):1271–1278.

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

Quinn N, Critchley P, Marsden CD. 1987. Young onset Parkinson's disease. Mov Disord 2(2):73–91.

Schrag A, Ben-Shlomo Y, Brown R, Marsden CD, Quinn N. 1998. Young-onset Parkinson's disease revisited—Clinical features, natural history, and mortality. Mov Disord 13(6):885–894.

Shimura H, Hattori N, Kubo S, Mizuno Y, Asakawa S, Minoshima S, Shimizu N, Iwai K, Chiba T, Tanaka K, Suzuki T. 2000. Familial Parkinson disease gene product, parkin is a ubiquitin-protein ligase. Nat Genet 25(3):302–305.

Tsuang DW, Dalan AM, Eugenio CJ, Poorkaj P, Limprasert P, La Spada AR, Steinbart EJ, Bird TD, Leverenz JB. 2002. Familial dementia with lewy bodies: A clinical and neuropathological study of 2 families. Arch Neurol 59(10):1622–1630.

West A, Periquet M, Lincoln S, Lucking CB, Nicholl D, Bonifati V, Rawal N, Gasser T, Lohmann E, Deleuze JF, Maraganore D, Levey A, Wood N, Durr A, Hardy J, Brice A, Farrer M. 2002. Complex relationship between Parkin mutations and Parkinson disease. Am J Med Genet 114(5):584–591.