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

Aida M. Bertoli-Avella, MD, PhD 1 José L. Giroud-Benitez, MD, 2 Ali Akyol, MD, 3 Egberto Barbosa, MD, 4 Onno Schaap, 1 Herma C. van der Linde, 1 Emilia Martignoni, MD, 5 Leonardo Lopiano, MD, 6 Paolo Lamberti, MD, 7 Emiliana Fincati, MD, 8 Angelo Antonini, MD, 9 Fabrizio Stocchi, MD, 10 Pasquale Montagna, MD, 11 Ferdinando Squitieri, MD, PhD, 12 Paolo Marini, MD, 13 Giovanni Abbruzzese, MD, 14 Giovanni Fabbrini, MD, 10 Roberto Marconi, MD, 15 Alessio Dalla Libera, MD, 16 Giorgio Trianni, MD, 17 Marco Guidi, MD, 18 Antonio De Gaetano, MD, 19 Gustavo Boff Maegawa, MD, 20 Antonino De Leo, MD, 21 Virgilio Gallai, MD, 22 Giulia de Rosa, MD, 23 Nicola Vanacore, MD, 24 Giuseppe Meco, MD, 10 Cornelia M. van Duijn, PhD, 1 Ben A. Oostra, PhD, 2
Peter Heutink, PhD, 25 Vincenzo Bonifati, MD, PhD, 1,10* and The Italian Parkinson Genetics Network †

1 Genetic-Epidemiologic Unit, Department of Clinical Genetics and Department of Epidemiology & Biostatistics, Erasmus MC Rotterdam, The Netherlands; 2 University Hospital Carlos J. Finlay, Havana, Cuba; 3 Department of Neurology, Adnan Menderes University, Aydin, Turkey; 4 Department of Neurology, University of São Paulo, São Paulo, Brazil; 5 Neurological Institute IRCCS Mondino, Pavia, and A. Avogadro University, Novara, Italy; 6 Department of Neuroscience, University of Turin, Turin, Italy; 7 Department of Neurology, University of Bari, Bari, Italy; 8 Department of Neurology, University of Verona, Verona, Italy; 9 Parkinson Institute, Istituti Clinici di Perfezionamento, Milan, Italy; 10 Department of Neurological Sciences, University La Sapienza, Rome, Italy; 11 Department of Neurology, University of Bologna, Bologna, Italy; 12 Neurogenetics Unit, IRCCS Neuromed, Pozzilli, Italy; 13 Department of Neurology, University of Florence, Florence, Italy; 14 Department of Neurosciences, Ophthalmology and Genetics, University of Genova, Genova, Italy; 15 Neurology Division, Hospital Misericordia, Grosseto, Italy; 16 Neurology Division, Hospital Boldrini, Thiene, Italy; 17 Neurology Division, Hospital of Casarano, Casarano, Italy; 18 Neurology Division, INRCA Institute, Ancona, Italy; 19 Neurology Division, Hospital of Castrovillari, Castrovillari, Italy; 20 Medical Genetics Service, Hospital de Clinicas, Porto Alegre, Brazil; 21 Neurology Division, Hospital Piemonte, Messina, Italy; 22 Department of Neurology, University of Perugia, Perugia, Italy; 23 Division of Neurology, Hospital of Ivera, Ivera, Italy; 24 National Centre of Epidemiology, National Institute for Health, Rome, Italy; 25 Section Medical Genomics, Department of Human Genetics and Department of Biological Psychology, VU University Medical Center, Amsterdam, The Netherlands

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, IVS11-3G, 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

*Correspondence to: Dr. Vincenzo Bonifati, Department Clinical Genetics, Erasmus MC Rotterdam, P.O. Box 1738, 3000 DR Rotterdam, The Netherlands. E-mail: v.bonifati@erasmusmc.nl
†A complete list of the Italian Parkinson Genetics Network members is presented in the Appendix.
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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), and mutations in three genes, parkin, DJ-1, and PINK1, have been identified. Parkin mutations vary from point mutations to complex rearrangements, including deletions and/or multiplications of complete exons. 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%). 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. 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. 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. Atypical clinic and genetic presentations, including pseudodominant inheritance 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. 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. Neurological examination was performed by neurologists with experience in movement disorders and included the Unified Parkinson’s Disease Rating Scale (UPDRS, Motor part) and Hoehn and Yahr scale 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.

**Molecular Studies**

**Haplotype Analysis.**

In the families compatible with autosomal recessive inheritance, we typed short tandem repeat (STR) markers from the PARK2/parkin, PARK6/PINK1, and PARK7/DJ-1 regions, using PCR with fluorescently labeled primers and an ABI 3100 automatic DNA analyzer, as detailed previously. 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. 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 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.

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 (Bio-Rad) 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 µl, 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. 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 (RP/β) using the following formula:

\[
RP/β = \frac{(CCT_{Parkin} - CCT_{β-globin})}{(PCT_{Parkin} - PCT_{β-globin})^2}
\]

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 χ² 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
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 polymorphisms 5'1239G>C (Val380Leu), 1281G>A (Asp394Asn), IVS2+2T>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

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### Table 1

**Phenotype description of the complete sample set and according to parkin genotype**

<table>
<thead>
<tr>
<th>Characteristics</th>
<th>Total sample set</th>
<th>Patients with parkin mutations</th>
<th>Patients without parkin mutations</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gender</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Male (%)</td>
<td>34 (57)</td>
<td>60</td>
<td>11 (48)</td>
</tr>
<tr>
<td>Age at onset, yr (range)</td>
<td>33 ± 11 (14–65)</td>
<td>60</td>
<td>28 ± 9 (15–44)</td>
</tr>
<tr>
<td>Disease duration, yr (range)</td>
<td>15 ± 9 (1–36)</td>
<td>59</td>
<td>20 ± 9 (6–36)</td>
</tr>
<tr>
<td>Age at examination, yr (range)</td>
<td>49 ± 10 (19–71)</td>
<td>59</td>
<td>49 ± 10 (32–70)</td>
</tr>
<tr>
<td>Symptoms and signs at onset</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Bradykinesia (%)</td>
<td>31 (54)</td>
<td>57</td>
<td>10 (48)</td>
</tr>
<tr>
<td>Resting tremor (%)</td>
<td>30 (53)</td>
<td>57</td>
<td>10 (48)</td>
</tr>
<tr>
<td>Asymmetry (%)</td>
<td>46 (79)</td>
<td>58</td>
<td>13 (62)</td>
</tr>
<tr>
<td>Dystonia (%)</td>
<td>7 (12)</td>
<td>57</td>
<td>3 (14)</td>
</tr>
<tr>
<td>Clinical signs at examination</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Bradykinesia (%)</td>
<td>55 (96)</td>
<td>57</td>
<td>21 (100)</td>
</tr>
<tr>
<td>Resting tremor (%)</td>
<td>37 (66)</td>
<td>56</td>
<td>14 (67)</td>
</tr>
<tr>
<td>Rigidity (%)</td>
<td>52 (91)</td>
<td>57</td>
<td>19 (90)</td>
</tr>
<tr>
<td>UPDRS off (range)</td>
<td>49 ± 21.2 (6–90)</td>
<td>24</td>
<td>41 ± 20.5 (6–70)</td>
</tr>
<tr>
<td>UPDRS on (range)</td>
<td>20 ± 11.0 (2–45)</td>
<td>42</td>
<td>20 ± 12.8 (2–43)</td>
</tr>
<tr>
<td>Hoehn &amp; Yahr off (range)</td>
<td>3.3 ± 0.9 (1–5)</td>
<td>30</td>
<td>2.9 ± 0.9 (1–4)</td>
</tr>
<tr>
<td>Hoehn &amp; Yahr on (range)</td>
<td>1.8 ± 0.7 (0–4)</td>
<td>48</td>
<td>1.9 ± 0.9 (0–4)</td>
</tr>
<tr>
<td>Treatment</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>With l-dopa (%)</td>
<td>46 (88)</td>
<td>52</td>
<td>17 (81)</td>
</tr>
<tr>
<td>Daily dose of l-dopa, mg (range)</td>
<td>556 ± 304 (100–1,250)</td>
<td>44</td>
<td>497 ± 337 (150–1,250)</td>
</tr>
<tr>
<td>Duration, mo. (range)</td>
<td>123 ± 92 (3–336)</td>
<td>36</td>
<td>139 ± 102 (8–290)</td>
</tr>
<tr>
<td>Other features (%)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>l-Dopa–induced dyskinesias</td>
<td>34 (79)</td>
<td>45</td>
<td>12 (75)</td>
</tr>
<tr>
<td>l-Dopa-induced motor fluctuations</td>
<td>33 (73)</td>
<td>43</td>
<td>10 (63)</td>
</tr>
</tbody>
</table>

*P = 0.02; bP = 0.005.

cP = 0.06; dP = 0.006 (the last two after adjustment for disease duration).

UPDRS, Unified Parkinson’s Disease Rating Scale.
shared haplotypes at the PARK2 locus (Fig. 1), without evidence of homozygosity.

The IVS11-3C \textsuperscript{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 ACAG/GAG to 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 \textsuperscript{A}, found in an Italian patient, was located further away from the splicing site. The computer programs predicted no affection 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. \textsuperscript{5,30}

**TABLE 2.**

Mutational screening of the parkin gene

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

*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.

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**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
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 \leq 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 $23 \pm 8$ years ($n = 7$) vs. $31 \pm 9$ years ($n = 16$) in the group with two exon deletions and $39 \pm 10$ 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, $22 \pm 10$ years, vs. $19 \pm 9$ and $13 \pm 8$ 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 ($41 \pm 20.5$ vs. $53 \pm 22$, $P = 0.057$) and Hoehn and Yahr scale measured in off status ($2.9 \pm 0.9$ vs. $3.4 \pm 0.9$, $P = 0.006$). 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. 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 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.

Semi-quantitative 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. 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.

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 that duplications.

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. Among our isolated patients, mutations were found in 67% of the patients with a disease onset ≤ 20 years old, in 14% and 6% of the patients with AAO between 21 and 30 years and ≥ 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.

Previous studies suggested that a single parkin mutation might sometimes cause EOP or represent a risk factor for late-onset PD. 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. Recently, a more severe disease status was reported in carriers of one missense mutation compared to carriers of two truncating mutations. Moreover, it has been suggested that missense mutations within the functional domains of the parkin protein led to earlier AAO. 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.

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APPENDIX

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Additional co-authors are: Susan Hsin Fen Chien, Aurelio Pimenta Dutra, Suely K. Nagahashi, Department of Neurology, University of São Paulo, São Paulo, Brazil; Laura Jardim, Carlos Rieder, Hospital de Clinicas de Porto Alegre, Brazil; Nefati Kiyioglu, Kobra Temocin, Hakar Ulucan, Adnan Menderes University, Aydın, Turkey.

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