Association Study of Parkin Gene Polymorphisms With Idiopathic Parkinson Disease

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Background: Previously, we detected linkage of idiopathic Parkinson disease (PD) to the region on chromosome 6 that contains the Parkin gene (D6S305; logarithm of odds score, 5.47) in families with at least one individual with age at onset younger than 40 years (families with early-onset disease). Further study demonstrated the presence of Parkin mutations in this data set. However, previous case-control studies have reported conflicting results regarding the role of more common Parkin polymorphisms as susceptibility alleles for idiopathic PD.

Objective: To investigate the association of 7 previously studied Parkin single-nucleotide polymorphisms (SNPs) throughout the promoter and most of the open reading frame with PD in a large cohort of patients with primarily late-onset PD.

Methods: One promoter, 3 intronic, and 3 exonic Parkin SNPs were genotyped in 1580 individuals belonging to 397 families, and their association with PD was evaluated using family-based association tests.

Results: No significant association ($P > .05$) between PD and any Parkin SNP allele or genotype was detected. Haplotype analysis and stratification by age at onset or family history also failed to produce significant results.

Conclusions: These results suggest that these common variants of Parkin are not associated with PD in white patients, although Parkin mutations are known to cause early- and late-onset PD.

Arch Neurol. 2003;60:975-980

PARKINSON DISEASE (PD) is the second most common human neurodegenerative disorder and is typically characterized by resting tremor, bradykinesia, and rigidity. The incidence and prevalence of PD increase with age, and thus, as the current population ages, the number of patients with PD will increase. Parkinson disease is a complex disease thought to result from the interaction of environmental and genetic factors. We completed a genomic screening of patients with idiopathic PD collected from 13 collaborating centers and found evidence in support of linkage to several genomic regions. Overall, there was little evidence for linkage on chromosome 6. However, when families were stratified by age at onset (AAO), significant evidence for linkage with a microsatellite lying in the Parkin gene (logarithm of odds score, 5.47 at marker D6S305) was detected in 18 families, each containing at least one individual with an AAO younger than 40 years. Parkin is an E2-dependent ubiquitin protein ligase known to carry pathogenic mutations in patients with autosomal recessive juvenile parkinsonism, but its role in idiopathic PD is still being explored. Subsequent analysis revealed the presence of 9 different Parkin mutations in 5% of our overall PD sample. We define mutations as genetic variants severe enough to cause an alteration of the protein function. Mutations are observed in affected individuals and possibly in their siblings but not in the general population.

This finding suggests that “normal” Parkin polymorphisms could act as susceptibility alleles for PD. Unlike mutations, these polymorphisms are common in the population. Polymorphisms are genetic variations present in both affected and unaffected individuals but may be associated with disease if the frequencies of the alleles or genotypes vary between these 2 groups of individuals. Previous studies have considered this hypothesis, but case-control association studies of Parkin single-nucleotide polymorphisms (SNPs) ($-258T>G$, Ser167Asn, Arg366Trp, Val380Leu, and Asp394Asn) were performed on small cohorts and led to conflicting results. To address this ques-
tion using previously analyzed SNPs, we studied the association of 1 promoter, 3 intronic, and 3 exonic Parkin polymorphisms with PD in a much larger family-based data set.

**METHODS**

**PATIENTS AND FAMILIES**

Data and blood from individuals with PD and their families were collected by the 13 centers of the Duke Center for Human Genetics (DCHG)/GlaxoSmithKline Parkinson Disease Genetics Collaboration and the DCHG Morris K. Udall Centers of Excellence for Parkinson’s Disease Research family ascertainment core. A standard clinical evaluation involves the Unified Parkinson’s Disease Rating Scale.8 Diagnosis of PD considers the presence of 3 cardinal signs: resting tremor, bradykinesia, and rigidity. All participating clinicians defined strict consensus clinical criteria before the ascertainment of families.1,9 Affected individuals possess at least 2 cardinal signs, no atypical features, and no other causes of parkinsonism. Unaffected participants demonstrated no signs of the disease, and unclear participants showed only 1 cardinal sign and/or atypical features. Individuals with a history of encephalitis, neuroleptic therapy within 1 year before diagnosis, evidence of normal pressure hydrocephalus, or a clinical course with unusual features suggestive of atypical or secondary parkinsonism were excluded from the study. To guarantee diagnostic consistency across sites, a clinical adjudication board consisting of a board-certified neurologist and a certified physician assistant reviewed the clinical data for all participants. All participants signed informed consents before blood and data collection. The study was approved by the local institutional review board. Both AAO and age at examination (AAE) were recorded, with AAO based on patient recollection of the AAO of the first cardinal sign of PD. All participants were white.

The families (N=397) used in this association study (Table 1) were drawn from 2 data sets ascertained for genetic studies of PD. The first data set consisted of singleton families (n=219), characterized by having only 1 sampled affected individual, with any number of unaffected family members. Eighteen of the singleton families have a reported family history of PD. The second data set consisted of multiplex families (n=178), comprising nuclear families (families with at least 2 sampled affected siblings and any number of unaffected individuals) or extended pedigrees (families with at least 2 sampled affected individuals who are not siblings or a parent-child pair, with any number of unaffected individuals). This data set includes families that were used in a previous genomic screening for PD.1

For analysis, the families were stratified by AAO and family history of PD. Early-onset families (n=52) were characterized by having at least one affected individual with an AAO younger than 40 years, whereas in late-onset families (n=345) all affected individuals have an AAO of 40 years or older. Unlike families with a history negative for PD (n=201), families with a history positive for PD (n=196) have 2 or more affected individuals who are first- to third-degree relatives.

**DATA EXTRACTION AND GENOTYPING**

DNA samples were prepared and stored by the Center for Human Genetics, Duke Institute of Genomic Science and Policy, DNA Bank Core.10 Genomic DNA was extracted from whole blood using the PUREGENE DNA Purification kit (Gentra Systems Inc, Minneapolis, Minn). In this protocol, the cells are lysed, the proteins are precipitated, and the DNA is pelleted and rehydrated. We genotyped 1 promoter (−258T>G), 3 exonic (Ser167Asn, Val380Leu, and Asp394Asn), and 3 intronic (IVS2+25T>C, IVS3−20C>T, and IVS7−35A>G) previously described Parkin SNPs.4,11-13 These SNPs (−258T>G−284.7 kb−IVS2+25T>C−242 kb−IVS3−20C>T−0.1 kb−Ser167Asn−631.7 kb−IVS7−35A>G−182.7 kb−Val380Leu−26.6 kb−Asp394Asn) cover the Parkin promoter and most of the Parkin genomic region. Intronic and exonic SNPs were genotyped using a modification of the gel-based oligonucleotide ligation assay14 as described by Martin et al,15 and the promoter SNP was genotyped using a TaqMan (Applied Biosystems, Foster City, Calif) allelic discrimination assay. The TaqMan polymerase chain reaction (PCR) amplification was performed in 5-μL reactions (30 ng of dried DNA, 1×TaqMan universal PCR master mix from Applied Biosystems, 900nM of each primer, 200nM of each probe) using the GeneAmp PCR system 9700 thermocyclers (Applied Biosystems) for a 40-cycle program (50°C for 2 minutes; 95°C for 10 minutes; 40×[95°C for 13 seconds and 60°C for 1 minute]). The fluorescence generated during the PCR amplification was read using the ABI Prism 7900HT sequence detection system and analyzed with the SDS software (both from Applied Biosystems). Table 2 gives the primers and probes used to genotype Parkin SNPs.

**STATISTICAL ANALYSES**

Single-locus tests for association were conducted using a likelihood ratio test (LRT) implemented in the computer program TRANSMIT and the pedigree disequilibrium test (PDT).16,17 These 2 tests are complementary tests of association that use different aspects of the data. The PDT directly compares allele frequencies between affected and unaffected siblings, whereas the LRT of TRANSMIT uses sibling genotypes to infer missing parental genotypes and then looks at parental transmissions to affected offspring only. The tests have different properties and thus both were used in this analysis. We used a version of the PDT based on the PDT-sum statistic.18 The robust variance estimator was used in the LRT to assure validity as a test of association in sibships of arbitrary size. The data set used for our association analyses consists of a few extended pedigrees; thus,

<table>
<thead>
<tr>
<th>Family Type</th>
<th>Families</th>
<th>Individuals</th>
<th>Affected</th>
<th>Unaffected</th>
<th>Unclear</th>
</tr>
</thead>
<tbody>
<tr>
<td>Singleton</td>
<td>219</td>
<td>656</td>
<td>247</td>
<td>365</td>
<td>44</td>
</tr>
<tr>
<td>Multiplex</td>
<td>178</td>
<td>924</td>
<td>360</td>
<td>507</td>
<td>57</td>
</tr>
<tr>
<td>Extended families</td>
<td>59</td>
<td>359</td>
<td>124</td>
<td>216</td>
<td>19</td>
</tr>
<tr>
<td>Nuclear families</td>
<td>119</td>
<td>565</td>
<td>236</td>
<td>291</td>
<td>38</td>
</tr>
<tr>
<td>Total</td>
<td>397</td>
<td>1580</td>
<td>667</td>
<td>872</td>
<td>101</td>
</tr>
</tbody>
</table>

*All multiplex families and 18 of the singleton families have a family history of Parkinson disease.*
we report the LRT analysis based on all nuclear families and a single nuclear family per extended pedigree. Analyses of association with haplotypes of SNPs were conducted using TRANSMIT. P values for a global test of significance were computed using the $\chi^2$ distribution with $h^{-1}df$, where $h$ is the number of distinct haplotypes observed ($h=2$ for single-locus tests). For the PDT analysis, individuals with a diagnosis of unclear are not used. The LRT uses the genotypes of unclear individuals only to aid in the inference of parental genotypes and haplotypes; the phenotype data on unclear and unaffected individuals are not used in the LRT.

In addition, a variation of the PDT, genotype-PDT, which assesses association among genotypes, rather than alleles, and risk of PD was conducted. To investigate the association of Parkin SNPs with AAO of PD, we used the variance components approach, considering both the model by Fulker et al and the orthogonal model implemented in the QTDT (Quantitative Transmission Disequilibrium Test) program. Because the variance components framework accounts for familial correlations, it is more appropriate to use than linear regression for multiplex families. Both models used an LRT to evaluate the association between AAO and alleles of the Parkin SNPs.

A single affected and unaffected individual were selected at random from each family for tests of Hardy-Weinberg disequilibrium (HWE) and linkage disequilibrium (LD) among markers. Analyses were conducted in the affected sample and unaffected sample separately, using a permutation test with 3200 permutations to estimate each $P$ value. For all statistical tests, we considered $P/11021 < 0.05$ a significant result.

### RESULTS

The total sample population in this study consists of 607 patients with PD (average±SD AAO, 62±13 years; range, 17-90 years; 59% male), 872 unaffected relatives (average±SD AAE, 67±12 years; range, 27-96 years; 45% male), and 101 unclear individuals (average±SD AAE, 71±11 years; range, 36-96 years; 48% male) from a total of 397 families. Table 1 presents the total number of individuals and families and the number of individuals by affection status in each family type. This sample does not include the 16 families previously found to carry Parkin mutations. The Figure depicts the location of the analyzed SNPs within the Parkin gene.
No evidence for deviation from HWE was detected in the affected or unaffected samples, with the exception of SNP 2 (P = .03) in affected individuals only. No significant LD was observed among SNPs, except for the intronic SNPs 2 and 3 (P = .01) in affected individuals, as well as SNPs P and 2 (P = .002) and SNPs 3 and 7 (P = .02) in unaffected individuals.

Statistical analyses were conducted to test the association of individual SNP alleles with PD. The estimated allele frequencies (Table 3) are in agreement with previous reports.6,12,23 No significant association with PD was observed for any of the Parkin polymorphisms using the LRT or the PDT analysis in the overall data set (Table 3). The allele frequencies in the patients were similar to the frequency estimates in the population (Table 3). We also did not find an association between genotypes and disease using the genotype-PDT (Table 3). A comparison of genotype frequencies in affected and unaffected siblings showed frequencies to be similar in the 2 groups (results not shown).

To further refine the analysis, families were stratified by AAO and family history of PD. Using both the LRT and PDT, no significant association for any of the Parkin SNPs was detected when the data were stratified by families with early- vs late-onset disease or by positive PD history vs negative PD history. Tests for association between AAO and SNP alleles using the QTDT program also failed to produce significant results (Table 3).

<table>
<thead>
<tr>
<th>Test</th>
<th>Allele</th>
<th>Population Frequency*</th>
<th>Case Frequency</th>
<th>LRT</th>
<th>PDT</th>
<th>Genotype-PDT</th>
<th>QTDT</th>
</tr>
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<tbody>
<tr>
<td>Single SNPs</td>
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<td></td>
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<tr>
<td>P</td>
<td>2 T</td>
<td>0.7976</td>
<td>0.8011</td>
<td>.71</td>
<td>.34</td>
<td>.498</td>
<td>.52</td>
</tr>
<tr>
<td>2</td>
<td>1 T</td>
<td>0.7759</td>
<td>0.7879</td>
<td>.91</td>
<td>.62</td>
<td>.64</td>
<td>.69</td>
</tr>
<tr>
<td>3</td>
<td>1 C</td>
<td>0.9148</td>
<td>0.9165</td>
<td>.45</td>
<td>.33</td>
<td>.42</td>
<td>.18</td>
</tr>
<tr>
<td>4</td>
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<td>0.9750</td>
<td>.24</td>
<td>.08</td>
<td>.08</td>
<td>Not tested</td>
</tr>
<tr>
<td>7</td>
<td>2 A</td>
<td>0.5623</td>
<td>0.5590</td>
<td>.33</td>
<td>.50</td>
<td>.41</td>
<td>.33</td>
</tr>
<tr>
<td>10</td>
<td>2 G</td>
<td>0.8408</td>
<td>0.8513</td>
<td>.16</td>
<td>.07</td>
<td>.24</td>
<td>.73</td>
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<td>11</td>
<td>2 G</td>
<td>0.9509</td>
<td>0.9527</td>
<td>.47</td>
<td>&gt; .99</td>
<td>&gt; .99</td>
<td>.19</td>
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<td>Seven SNPs, haplotype</td>
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<td>P2.3.4.7.10.11‡</td>
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<td>0.0348</td>
<td>0.0283</td>
<td>.10</td>
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<td>2.1.1.2.2.1.2</td>
<td>0.0658</td>
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<td>NA</td>
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<td>1.1.1.2.1.2.2</td>
<td>0.0434</td>
<td>0.0415</td>
<td>&gt; .99</td>
<td>NA</td>
<td>NA</td>
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<td>0.2066</td>
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<td>NA</td>
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<td>2.2.1.2.1.2.2</td>
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<td>.82</td>
<td>NA</td>
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<tr>
<td>1.1.1.2.2.2.2</td>
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<td>NA</td>
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<td>2.1.1.2.2.2.2</td>
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<td>Global haplotype test</td>
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</table>

Abbreviations: LRT, likelihood ratio test; NA, not applicable; PDT, pedigree disequilibrium test; QTDT, Quantitative Transmission Disequilibrium Test; SNP, single-nucleotide polymorphism.

*Population frequency indicates the maximum likelihood estimate for allele frequencies in parents of sampled affected individuals for single SNPs and maximum likelihood estimate for 6 SNP haplotype frequencies in parents of sampled affected individuals.

†Orthogonal model was performed to test the association for age of onset in a variance component. This model allows for families of any size with or without parental genotypes. For SNP 4, the test was not performed due to low heterozygosity.

‡Only the haplotypes with an observed frequency larger than 2% are shown.

As an example, Table 3 presents the population and patient haplotype frequencies and the P values for association with the most common (>2% frequency) Parkin haplotypes with all 7 SNPs. Notably, only 9 common (>2% frequency) haplotypes were observed, and their frequencies are close to those expected under linkage equilibrium, which is consistent with our finding that there was little LD between SNPs. Because many of the SNPs had an allele of low frequency, many of the possible haplotypes were of low frequency, even with linkage equilibrium, and thus were not included in the analysis.

The results presented in this study suggest that none of these 7 Parkin SNPs, either independently or jointly, confer increased susceptibility or protection to developing idiopathic PD in white patients, nor are they in strong LD with a common susceptibility allele. We found no evidence for genetic association using single-locus or haplotype family–based association tests, even when the overall data set was stratified by AAO or family history.

Previous Parkin association studies3-7,23-25 reported conflicting results. Oliveri et al23 found no differences in allele and genotype frequencies at SNPs 4 and 10 between patients with early-onset disease (which they defined as AAO >45 years) and controls. On the other hand, Satoh and Kuroda3 reported that the frequency of the SNP 4 heterozygotes was significantly higher in patients with sporadic PD than in age-matched controls, and Klein et al25 detected a significant difference in allele frequencies for the SNP 10 between patients with early-onset disease (which they defined as AAO <50 years) and con-
controls. Although our results did not reach statistical significance, they pointed in the same direction, since we obtained P values of .07 in the overall sample for SNPs 4 and 10 with PDT and for SNP 4 using genotype-PDT. Furthermore, Mata and colleagues reported that genetic variation in the Parkin gene and its promoter does not contribute to risk of developing PD, whereas West and colleagues found that the –258T>G SNP is associated with idiopathic PD and that different alleles have different nuclear protein binding affinities and promoter activities. Discrepancies between these studies and the present report may derive from a type I error (false-positive result), different patient ascertainment methods, or population-specific effects. The present study also differs from the previous ones in the large number of individuals involved and in the use of family controls rather than general population controls.

None of these 7 Parkin SNPs were in significant LD in the overall data set, with the exception of the intronic SNPs 2 and 3 in affected individuals, as well as SNPs P and 2, and SNPs 3 and 7 in unaffected individuals. This lack of LD can also be appreciated in the diversity of haplotypes observed when the 7 SNPs were combined. Although Parkin is an extremely large gene and these SNPs cover a 1.368-Mb genomic region, some LD between SNPs was expected, especially among exonic (SNPs 4, 10, and 11) or closely spaced (SNPs 3 and 4, and SNPs 10 and 11) polymorphisms. A sequence alignment of the human, mouse, rat, and Drosophila Parkin orthologues (GenBank accession Nos. AB009973, AF250293, AF257234, and AY093423, respectively) reveals that the Ser167 (SNP 4) and Val380 (SNP 10) amino acids are not conserved among different species, whereas the Asp394 (SNP 11) residue is completely conserved. These data suggest that Parkin is a gene with a high recombination or mutation rate, which is in agreement with the large number of known Parkin mutations. The lack of LD between SNPs 2 and 3, only 108 base pairs apart, was also unexpected. This finding is interesting given that most known deletions in the Parkin gene lie in this region. These data, coupled with the lack of LD shown herein, suggest an unstable region.

Significant deviation from the HWE was found with SNP 2 in the affected individuals only. It has been suggested that lack of HWE indicates the presence of a susceptibility allele or one that is in LD with such an SNP, but this is unlikely since we found no association of this SNP with PD using the PDT or the LRT. It is also possible that this deviation from HWE is not related to disease but indicates true departure from HWE in the population and that differences between the results in affected and unaffected samples represent statistical variation. Another possibility is that the result in the affected sample is false positive, which may be expected given the many statistical tests performed. Finally, departure from HWE could be a result of genotyping error, but extensive quality control measures were undertaken to reduce that error.

No evidence for association was found in families with early-onset PD, although we detected linkage in the families with early-onset disease in our genomic screening. Subsequent screening of PD families showed that several of the families with early-onset disease carry Parkin mutations on different genetic backgrounds. This suggests that the linkage peak in this subset of families is a result of these mutations and not of a more general association, explaining why no association could be detected.

Accepted for publication December 17, 2002.

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Author contributions: Study concept and design (Drs Oliveira, W. K. Scott, Pericak-Vance, Vance, and Martin and Mr Booze); acquisition of data (Drs Oliveira, W. K. Scott, Nance, Watts, Hubble, Koller, Lyons, Pahwa, Stern, Hiner, Jankovic, Ondo, Allen, B. L. Scott, Goetz, Small, Mastaglia, Middleton, Haines, Pericak-Vance, and Vance and Messrs Stajich, Booze, and Reaves); analysis and interpretation of data (Drs Oliveira, W. K. Scott, Zhang, Pericak-Vance, Vance, and Martin and Mr Booze); drafting of the manuscript (Drs Oliveira, W. K. Scott, Zhang, Pericak-Vance, and Vance); critical revision of the manuscript for important intellectual content (Drs Oliveira, W. K. Scott, Nance, Watts, Hubble, Koller, Lyons, Pahwa, Stern, Hiner, Jankovic, Ondo, Allen, B. L. Scott, Goetz, Small, Mastaglia, Zhang, Middleton, Haines, Pericak-Vance, Vance, and Martin and Messrs Stajich, Booze, and Reaves); statistical expertise (Drs W. K. Scott, Zhang, Pericak-Vance, and Martin); obtained funding (Drs W. K. Scott, Pericak-Vance, Vance, and Martin); administrative, technical, and material support (Mr Booze and Drs Pericak-Vance and Vance); study supervision (Drs Oliveira, W. K. Scott, Pericak-Vance, and Martin and Mr Booze).

This study was supported in part by the Morris K. Udall Centers of Excellence for Parkinson's Disease Research grant 5 P50 NS39764-03. Additional funding was received from the National Institute on Aging (IR01-AG-20135-01), Bethesda, Md, and GlaxoSmithKline Inc, Research Triangle Park, NC. Dr Oliveira receives funding from a post-doctoral fellowship (BPD/5733/2001) granted by the Por-
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