

A comprehensive analysis of deletions, multiplications, and copy number variations in *PARK2*

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ABSTRACT

Objectives: To perform a comprehensive population genetic study of *PARK2*. *PARK2* mutations are associated with juvenile parkinsonism, Alzheimer disease, cancer, leprosy, and diabetes mellitus, yet ironically, there has been no comprehensive study of *PARK2* in control subjects; and to resolve controversial association of *PARK2* heterozygous mutations with Parkinson disease (PD) in a well-powered study.

Methods: We studied 1,686 control subjects (mean age 66.1 ± 13.1 years) and 2,091 patients with PD (mean onset age 58.3 ± 12.1 years). We tested for *PARK2* deletions/multiplications/copy number variations (CNV) using semiquantitative PCR and multiplex ligation-dependent probe amplification, and validated the mutations by real-time quantitative PCR. Subjects were tested for point mutations previously. Association with PD was tested as *PARK2* main effect, and in combination with known PD risk factors: *SNCA*, *MAPT*, *APOE*, smoking, and coffee intake.

Results: A total of 0.95% of control subjects and 0.86% of patients carried a heterozygous CNV mutation. CNV mutations found in 16 control subjects were all in exons 1–4, sparing exons that encode functionally critical protein domains. Thirteen patients had 2 CNV mutations, 5 had 1 CNV and 1 point mutation, and 18 had 1 CNV mutation. Mutations found in patients spanned exons 2–9. In whites, having 1 CNV was not associated with increased risk (odds ratio 1.05, $p = 0.89$) or earlier onset of PD (64.7 ± 8.6 heterozygous vs 58.5 ± 11.8 normal).

Conclusions: This comprehensive population genetic study in control subjects fills the void for a *PARK2* reference dataset. There is no compelling evidence for association of heterozygous *PARK2* mutations, by themselves or in combination with known risk factors, with PD. **Neurology**[®] 2010;75:1189–1194

GLOSSARY

ARJP = autosomal recessive juvenile parkinsonism; **CI** = confidence interval; **CNV** = copy number variation; **MAP** = moving average plots; **MLPA** = multiplex ligation-dependent probe amplification; **NGRC** = NeuroGenetics Research Consortium; **OR** = odds ratio; **PD** = Parkinson disease.

PARK2, a large gene in a fragile site on chromosome 6q25.2-q27,¹ encodes Parkin, an E3 ubiquitin-protein ligase. *PARK2* is also a tumor suppressor gene.^{2,3} Originally discovered as a cause of autosomal recessive juvenile parkinsonism (ARJP),⁴ *PARK2* has subsequently been linked to cancer,^{2,3} leprosy,⁵ autism,⁶ type 2 diabetes mellitus,⁷ and Alzheimer disease.⁸ Despite increasing appreciation for the potential involvement of *PARK2* in common disorders of diverse origins, there has not been a systematic large study of *PARK2* in control subjects. Our first

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aim was to conduct a population genetic study of *PARK2* and establish a reference dataset for the increasing number of *PARK2*-disease associations that are emerging from genome-wide association studies.

PARK2 is a molecular diagnostic test for parkinsonism. The majority of positive results are heterozygous and difficult for clinicians to interpret because whether having one mutation can cause, increase risk, or accelerate onset of Parkinson disease (PD) is unknown.⁹⁻²¹ The second aim of our study was to determine, conclusively, if heterozygous mutations are associated with PD. We designed this study specifically to address this question, and to that end, amassed a large sample size to ensure analytic power, analyzed the coding regions for all types of variations, and importantly, used the same rigorous mutation analysis and validation methods in control subjects as in patients. In the first phase, we established that heterozygous point mutations are as frequent in control subjects as in patients with PD.²² In this final phase, we present a comprehensive analysis of deletions/multiplications/copy number variations (CNV).

METHODS Subjects included 3,777 genetically unrelated individuals (1,686 control subjects and 2,091 patients with PD) from the NeuroGenetics Research Consortium (NGRC). Control subjects consisted of 1,644 white, 13 black, 11 Hispanic, 5 Asian, 4 Native American, and 9 other race/ethnicity, ages 25–99 years at blood draw (mean 66.1 ± 13.1 years), and 43.8% were men. Patients with PD consisted of 1,968 white, 18 black, 31 Hispanic, 29 Asian, 9 Native American, 2 Pacific Islander, and 34 other/unknown. They were diagnosed by a movement disorder neurologist according to the modified UK PD Brain Bank criteria²³ at one of 8 NGRC clinics in Oregon, Washington, New York, and Georgia. Patients were 8–93 years old at disease onset (58.3 ± 12.1) and 21–96 years old at blood draw (mean 67.3 ± 10.7 years), 67.8%

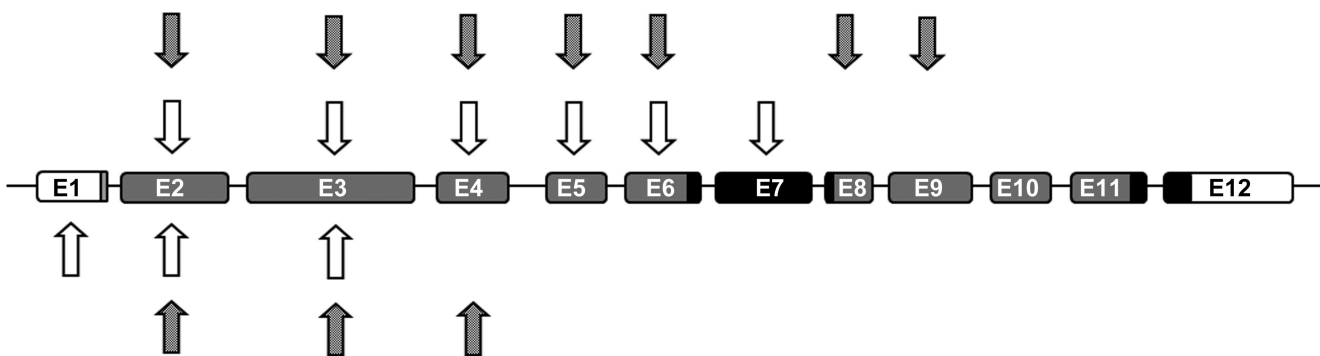
were men, and 22.7% had a positive family history (first-degree or second-degree relative with PD).

Standard protocol approvals, registrations, and patient consents. The study was approved by the Institutional Review Boards of participating institutions. Informed consent was obtained from all subjects.

Molecular analysis. CNV genotyping was completed for 2,091 patients and 1,686 controls using high-molecular-weight genomic DNA from blood. Genotyping was performed in phases over 7 years (details in appendix e-1 on the *Neurology*[®] Web site at www.neurology.org). In 2004, we published on 39 patients with young-onset PD.²⁴ While performing the large-scale sequencing (2004–2007), we identified 40 subjects with rare sequence variants and genotyped them for CNVs.²² The large-scale CNV analysis started with a modified semiquantitative PCR protocol²⁵ that was used for 252 patients and 299 control subjects. With the advent of PD-specific multiplex ligation-dependent probe amplification (MLPA),^{26,27} we switched to the SALSA MLPA kit P052B from MRC-Holland (Amsterdam, the Netherlands) for 1,956 patients and 1,477 control subjects. We used more conservative measures than recommended by the MLPA manufacturer and were able to establish false-positive and false-negative rates (appendix e-1). We used real-time qPCR to verify all abnormal and equivocal findings from MLPA and semiquantitative PCR. CNV carriers were sequenced for all exons to detect point mutations. Finally, using DNA from relatives, we established phase unequivocally for 39 of 52 CNV carriers.

Data analysis. Logistic regression, χ^2 tests, and Fisher exact tests were used to test frequencies and estimate odds ratios (ORs) and corresponding 95% confidence intervals (95% CI). Age at onset was tested using Kaplan-Meier survival analysis, log-rank statistics, and analysis of variance. Data were adjusted for recruitment site, gender, age at blood draw, cigarette smoking,²⁸ caffeinated coffee consumption,²⁸ and PD susceptibility genotypes at *SNCA* REP1,²⁹ *SNCA* 5' promoter polymorphism rs2619364,³⁰ *MAPT* H1/H2 haplotypes,³¹ and the *APOE* polymorphism.³² We tested for *PARK2* × environment (smoking and coffee) and *PARK2* × genotype (*SNCA*, *MAPT*, *APOE*) interaction using likelihood ratio test statistics. We selected the most significant factors using backward variable selection criteria. The model goodness-of-fit was tested using Hosmer and Lemeshow test statistics. Frequencies of CNVs in patients and control subjects were visualized as a function of age using moving average plots (MAP).³³

Figure 1 Location of *PARK2* copy number variations



Deletions (hatched arrows) and multiplications (open arrows) detected among 2,091 patients (top) and 1,686 control subjects (bottom). The *PARK2* coding region is shown in gray; the 2 ring domains are shown in black. Introns not drawn to scale. E = exon.

RESULTS Reference data. Reference data are detailed in table e-1 and figure 1. Sixteen of 1,686 control subjects had a CNV, yielding a heterozygous CNV carrier frequency of 0.95% (95% CI 0.48–1.42). None of the carriers was homozygous or compound heterozygous. The mutations included deletions and multiplications occurring predominantly in exons 2, 3, and 4. One control subject had a mutation in exon 1. No CNVs were detected in exons 5–12.

Parkinson study. The Parkinson study is described in table e-2 and figure 1. Among the 2,091 patients with PD, 13 had 2 CNVs, 5 had 1 CNV and 1 point mutation, and 18 had only 1 CNV. The mutations included deletions and multiplications in exons 2–9.

No mutations were found in exons 1 or 10–12. All subjects with 2 *PARK2* mutations had developed PD prior to age 55, which is consistent with autosomal recessive mutations causing young-onset PD. Among 171 patients with onset ≤ 40 years, 14 had 2 *PARK2* mutations (Parkin disease) and none of the other 296 was heterozygous.

The question that we aimed to resolve was whether heterozygous mutations increase the risk or accelerate onset age of PD. Heterozygous CNV carrier frequency in patients was 0.86% (95% CI 0.46–1.26) as compared to 0.95% (95% CI 0.48–1.42) in control subjects. For the following analyses, we excluded subjects with mutations in *LRRK2*, *SNCA*, or *SCA2* and nonwhites; the sample included 1,937 patients and 1,642 control subjects.

A striking feature of recessive *PARK2* disease is the very early onset. We therefore questioned if having one mutation might accelerate onset age in common forms of PD. Mean age at onset was not earlier in *PARK2* heterozygotes (64.7 ± 8.6 years) than in patients lacking a *PARK2* CNV mutation (58.5 ± 11.8 ; table 1). Kaplan-Meier-generated age at onset distributions revealed a highly significant age at onset effect ($p = 1 \times 10^{-40}$), which stemmed from ~ 30 years earlier onset for patients with 2 mutations (figure 2). The Kaplan-Meier age at onset distribution for heterozygotes was not significantly different from

Table 1 Association of *PARK2* CNV heterozygous mutations with PD in white individuals^a

<i>PARK2</i> main effect	OR	95% CI	p
Crude OR	1.05	0.50-2.19	0.89
Adjusted for <i>MAPT</i> , <i>SNCA</i> , <i>APOE</i> , coffee, smoking, site, gender, age	1.14	0.36-3.63	0.82
Familial PD adjusted for site, gender, age	0.99	0.28-3.56	0.99
Interaction	χ^2	df	p
<i>PARK2</i> \times gene	4.11	5	0.53
<i>PARK2</i> \times environment	1.15	2	0.56
<i>PARK2</i> \times gene and <i>PARK2</i> \times environment	5.00	8	0.76
Stepwise regression ^b	OR	95% CI	p
<i>PARK2</i> CNV 1 vs 0	1.14	0.36-3.63	0.82
<i>MAPT</i> H1/H1 vs H2 carrier	1.51	1.24-1.83	<0.001
<i>SNCA</i> 5' promoter rs2619364 TT vs TC	0.75	0.61-0.91	0.005
Coffee low vs high ^c	0.69	0.56-0.84	0.0002
Smoking ever vs never ^d	0.81	0.66-0.99	0.038
Gender female vs male	0.30	0.25-0.37	<0.001
Cox proportional hazard ^e	HR	95% CI	p
No. of <i>PARK2</i> mutations = 0	Ref.	Ref.	Ref.
No. of <i>PARK2</i> mutations = 1	0.98	0.60-1.60	0.92
No. of <i>PARK2</i> mutations = 2	26.68	15.52-45.86	1×10^{-32}
Analysis of variance: age at onset	Mean \pm SD	Range	p ^f
No. of <i>PARK2</i> mutations = 0	58.5 ± 11.8	14-93	3×10^{-14}
No. of <i>PARK2</i> mutations = 1	64.7 ± 8.6	46-75	
No. of <i>PARK2</i> mutations = 2	34.2 ± 10.4	8-53	

Abbreviations: CI = confidence interval; CNV = copy number variation; HR = hazard ratio; OR = odds ratio; PD = Parkinson disease.

^a Individuals with 2 CNV mutations or a combination of 1 CNV mutation and a rare (minor allele frequency <0.01) nonsynonymous point mutation were excluded.

^b Data shown only for *PARK2* and significant variables (i.e., those that did not fall out of the regression).

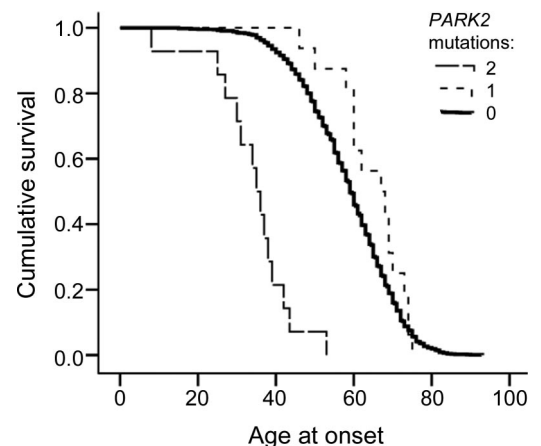
^c Caffeinated coffee consumption low defined as at or below the median consumption of control subjects, and high as above the median of control subjects.

^d Ever defined as smoking at least 100 cigarettes in lifetime.

^e Hazard ratios adjusted for recruitment site and gender.

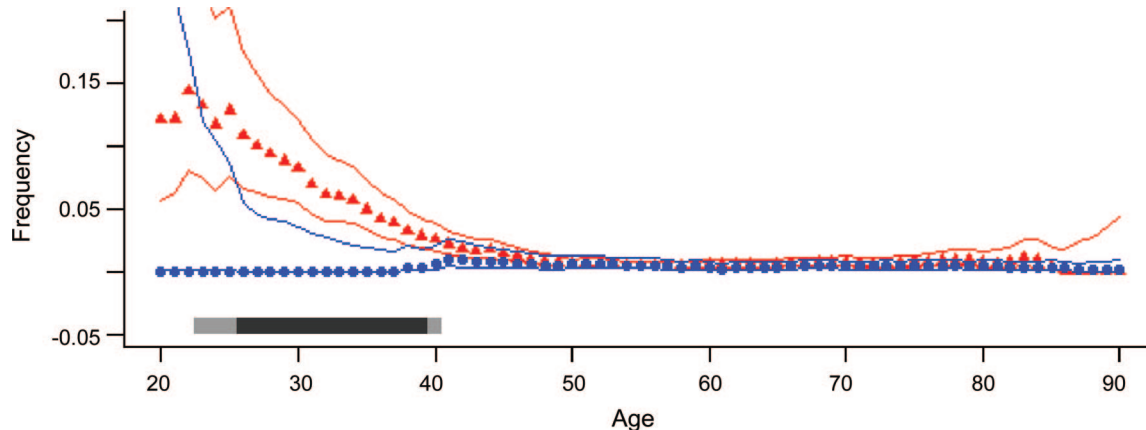
^f A single p value represents the significance of the difference between the 3 groups analyzed simultaneously.

Figure 2 Age at onset of Parkinson disease as a function of number of *PARK2* mutations



Patients with 2 (long dashed line), 1 (short dashed line), and 0 (solid line) *PARK2* mutations. Rare nonsynonymous sequence variants were considered to be a mutation only when found in an individual carrying a copy number variation (CNV). Age at onset in heterozygous *PARK2* patients is not earlier than in patients lacking a *PARK2* CNV mutation, as opposed to patients with compound mutations who have substantially earlier onset. The graph was generated using Kaplan-Meier survival analysis and the differences were tested using log-rank statistics. Overall, $p = 1 \times 10^{-40}$, which is driven by compound mutation carriers in recessive young-onset disease.

Figure 3 Moving average plots of *PARK2* mutation frequency as a function of age



Moving average frequency of *PARK2* mutations was estimated as a function of age at onset in patients (red triangles) vs age at blood draw in controls (blue circles) with 95% central posterior probabilities (red and blue lines). Plots show that *PARK2* mutation frequency is very high in young-onset Parkinson disease (PD), declines with increasing age at onset, and by age 45 and thereafter, the mutation frequency in PD and control subjects are completely superimposed. Gray bars at the bottom of the graph show the statistical significance of the difference between patients and control subjects, calculated in the moving window (light gray $\geq 95\%$ probability, dark gray $\geq 99\%$ probability). Due to few numbers at the extremes, subjects with ages ≤ 20 or ≥ 90 years were merged.

nonmutation carriers; in fact, heterozygotes appeared to have slightly delayed onset age (also reflected in mean onset ages), which is contrary to the hypothesis that *PARK2* accelerates PD onset.

To assess *PARK2* heterozygosity in relation to risk of PD, we included all patients and control subjects, regardless of age at onset or family history, and compared those with one mutation to those without. Fourteen subjects with compound *PARK2* mutations were excluded. The sample included 1,923 unrelated white patients and 1,642 unrelated white control subjects. We found no evidence ($p = 0.89$) for association of *PARK2* heterozygosity with PD risk in the overall sample (OR 1.05, 95% CI 0.50–2.19; table 1) or in patients with positive family history ($n = 423$, OR 0.99, 95% CI 0.28–3.56; table 1).

We hypothesized that *PARK2* heterozygosity might increase PD risk if patients also had a high-risk genotype at *MAPT*, *SNCA*, or *APOE*, did not smoke, did not consume large amounts of caffeinated beverages, were male, or were at advanced age. Adjusting for these factors in the model did not change the outcome (OR 1.14, $p = 0.82$; table 1). We tested for interaction between *PARK2* and the aforementioned genes and exposures, and found none ($p = 0.53$ – 0.76). We performed stepwise and backward regression, which yielded similar results. We detected significant effects on PD risk for every factor known to be associated with PD, but not for *PARK2* heterozygosity. We performed numerous iterative subanalyses to search for evidence that might be hidden in the type of mutation (deletion vs multiplication), exon location (exon 2 vs 3 vs 4, for example), and mutation phase, where we assigned and analyzed seemingly contiguous deletions/multiplications once

as heterozygous and once as compound (data not shown). We did not find any compelling evidence for association with PD.

The MAP (figure 3) captures the results at a glance: mutation frequency starts very high in young-onset PD, declines sharply with increasing age at onset until it meets the control frequency at around age 45, and from age 45 until 90, stays completely superimposed on control subjects.

DISCUSSION Currently, there exists no population genetic study of *PARK2* in the literature. The present study fills this void. We demonstrated that *PARK2* deletions, multiplications, and CNVs (this report), and rare sequence variants including point mutations (reported previously),²² are not exclusive to disease populations. We found CNVs in $\sim 1\%$ and point mutations in $\sim 3\%$ of control subjects. Heterozygous CNV mutations in exons 2–4 are common and well-tolerated. However, we did not find any CNVs in exons 5–12 in control subjects, which include the coding region for the highly conserved functional domains of Parkin. Mutations that affect these regions may be deleterious and hence rare or absent in healthy individuals. Alterations in these exons have been observed in ARJP as recessive germline mutations, and in malignancies as heterozygous somatic mutations. None of the 1,686 control subjects was homozygous or compound heterozygous, which implies biallelic mutations are pathogenic. This is certainly the case for ARJP. We do not know, however, if recessive genotypes can cause disorders other than ARJP.

The second component of the study, the role of *PARK2* in common PD, addressed a much-debated and extensively published controversy.^{9–21} Studies

have reported heterozygous mutations in patients that were absent in controls, suggesting *PARK2* heterozygosity is a risk factor for PD.^{10-14,16,17} However, not all studies found a higher frequency in patients than in controls.²⁰ Although the sampling of patients varied across studies (early-onset, late-onset, familial PD), the frequency of *PARK2* mutations in our patients with PD, when subgrouped to match each study, are generally in line with most prior studies. The main difference between our data and most other published datasets is in the controls. The frequency of *PARK2* mutation in our controls is higher than most, but is in line with Lincoln et al.²⁰ and Bruggemann et al.,²¹ who, like us, genotyped controls comprehensively. Most other studies performed detailed genotyping in patients and screened control subjects only for the mutations found in patients; thus, we suspect, missed the mutations that might have been present in controls but not in patients. The 3 studies that genotyped controls comprehensively had sample sizes of 192,²⁰ 356,²¹ and 1,686 (this study), and all 3 report 3%–4% of controls carrying heterozygous *PARK2* mutations. A study of familial PD, however, found no CNV mutations in 263 control subjects, which was significant compared to their patient population.¹⁴ Our study had 99% power to detect the effect size reported for familial PD,¹⁴ and ~90% power to detect a minimum OR of 1.5 overall; yet our OR and *p* values were ~1. The varied results of published data may be consolidated as follows when 2 key variables are considered: age and genotyping method. 1) *PARK2* mutation frequency is high in young-onset PD and drops sharply with increasing onset age, as clearly shown in figure 3. 2) Having *PARK2* mutations on both chromosomes causes disease, which is well-established. 3) Having one mutation occurs at a low frequency (<5%) in both patients and control subjects, and is detectable only when the sample size is large and rigorous genotyping methods are used.

There are caveats to our study. Results cannot be generalized to nonwhite populations, nor do they speak to subclinical disease that may be present in heterozygotes.¹⁸ Interaction tests had low power. It is possible that a single *PARK2* mutation is a “silent” etiologic factor that manifests clinically in combination with as yet unknown PD triggers.

AUTHOR CONTRIBUTIONS

Statistical analysis was conducted by Dr. D.M. Kay and Dr. T.H. Hamza.

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DISCLOSURE

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