

ORIGINAL ARTICLE

Comprehensive mutational analysis of *LRRK2* reveals variants supporting association with autosomal dominant Parkinson's disease

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Parkinson's disease (PD) is a neurodegenerative disorder characterized by neurodegeneration, most notably of dopaminergic neurons in the substantia nigra. To date, six causative genes have been identified including *LRRK2*, whose mutations are the most frequent in autosomal dominant PD (Ad-PD). We conducted a comprehensive mutational analysis of *LRRK2* in 30 Ad-PD (11 Japanese and 19 Caucasian) families employing a DNA microarray-based resequencing system and direct nucleotide sequence analysis, and identified 23 variants including two known mutations, p.G2019S and p.I1371V, in three Caucasian families and one Caucasian family, respectively, a novel putative pathogenic mutation, p.N1221K, in one Japanese family, and a known nonsynonymous variant, p.G2385R, in two Japanese families. Detailed analysis of the frequency of p.G2385R among 100 Japanese Ad-PD, 73 sporadic PD (sPD) and 238 controls revealed that the frequency of the p.G2385R variant was significantly higher in Ad-PD than in controls (allele frequency, 9.0 vs 2.1%) ($\chi^2=16.32$, $P=5.34\times 10^{-5}$). The p.G2385R variant, however, did not show complete cosegregation with PD. In addition, the frequency of p.G2385R was also higher in sPD than in controls, although not significant (allele frequency, 3.4 vs 2.1%) ($\chi^2=0.76$, $P=0.38$). These observations support the possibility that p.G2385R is associated with an increased risk of PD.

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INTRODUCTION

Parkinson's disease (PD) is a neurodegenerative disorder characterized by rigidity, tremor, akinesia and postural instability. Its pathological features include the progressive degeneration of dopaminergic neurons primarily in the substantia nigra, with Lewy bodies as the pathological hallmark. Although the majority of PD patients have sporadic PD (sPD), various forms of familial PD (FPD) have been recognized. Recent advances in the search for genes causing PD have contributed to a better understanding of the molecular pathophysiology of PD. To date, six genes (*SNCA*, *PARK2*, *DJ1*, *PINK1*, *UCHL1* and *LRRK2*) have been identified as genes causing FPD.^{1–6} The genetic heterogeneity necessitates a comprehensive mutational analysis of these genes for not only molecular diagnosis but also studies of the molecular epidemiology of FPD.

Among the genes causing FPD, *LRRK2* is of particular interest, because *LRRK2* is by far the most common cause of autosomal

dominant PD (Ad-PD). Approximately 4% of Ad-PD patients and 1% of sPD patients have been reported to harbor causative mutations of *LRRK2*, the most frequent of which is the mutation substituting serine for glycine at codon 2019 (p.G2019S).⁷ It is mandatory to carry out mutational analysis of *LRRK2* to conduct molecular diagnosis and to investigate the molecular epidemiology of Ad-PD. Analysis of the entire 51 exons of *LRRK2* by the conventional direct nucleotide sequencing method, however, is very laborious. Therefore, the majority of previous studies have focused on particular exons for mutational analysis,^{8–12} making it difficult to obtain accurate data on the molecular epidemiology of Ad-PD caused by *LRRK2*.

We herein applied a DNA microarray-based resequencing system to the comprehensive mutational analysis of all *LRRK2* exons in 30 families with Ad-PD from two cohorts with different ethnic backgrounds.

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MATERIALS AND METHODS

Subjects

To directly compare the *LRRK2* epidemiologies across ethnic groups in two similarly ascertained cohorts, we conducted a comprehensive resequencing analysis of *LRRK2* focusing on 11 Japanese and 19 Caucasian families with Ad-PD employing resequencing DNA microarrays (Table 1a). These families were diagnosed as having Ad-PD when their pedigree members in at least two generations were diagnosed as having PD. Two Caucasian probands were assessed both by the direct sequencing approach and by resequencing microarray analysis.¹³ To evaluate the molecular epidemiology of *LRRK2* variants identified by the resequencing of *LRRK2*, DNA samples from index patients from 89 Ad-PD families and 73 sPD patients, and samples from 233 Japanese normal controls were further analyzed (Table 1b). All the genomic DNA samples were obtained with the written informed consent of the subjects, and this research project was approved by the Institutional Review Board of the University of Tokyo.

Mutational analysis

The mutational analysis of all 51 *LRRK2* exons was accomplished using the DNA microarray-based resequencing system as described elsewhere.^{14,15} Briefly, specific PCR was conducted for each exon using previously reported primers.⁶ After quantification of the PCR products, they were pooled equimolarly, fragmented with DNase I, labeled with biotin, hybridized to microarrays, stained with streptavidin-phycoerythrin, washed and scanned. Direct nucleotide sequence analysis was also conducted using an automated DNA sequencer and BigDye Terminator ver. 3.1 (Applied Biosystems, Foster City, CA, USA).

RESULTS

Mutational analysis of *LRRK2* in 30 Ad-PD families

Twenty-three variants were identified by the resequencing of the 51 exons and the splice sites of *LRRK2* in 30 Ad-PD families (Table 2). Of the 10 nonsynonymous variants identified in the 11 Japanese Ad-PD families, two novel variants (p.N1221K and p.N1320S), both of which were identified in one family, respectively, and the previously reported nonsynonymous variant p.G2385R, which was identified in two families, were of interest. Of the 8 nonsynonymous variants identified in the 19 Caucasian Ad-PD

families, the previously known mutation p.G2019S, which was identified in three families, and two previously reported nonsynonymous variant p.R1514Q and p.I1371V, both of which were identified in one family, respectively, were of interest. The remaining seven nonsynonymous variants were previously been registered in the dbSNP database (<http://www.ncbi.nlm.nih.gov/SNP/index.html>) and not considered as mutations causing Ad-PD.

The two novel nonsynonymous variants found in the Japanese Ad-PD families are a variant substituting lysine for asparagine at codon 1221 (p.N1221K) (Figure 1a) and that substituting serine for arginine at codon 1320 (p.N1320S). p.N1221K was located in a highly conserved region (Figure 1b) and not found in 233 controls (466 chromosomes). The index patient with p.N1221K developed levodopa-responsive parkinsonism including asymmetric rigidity and postural instability at age 47. The other novel nonsynonymous variant p.N1320S was found in one of the 11 Japanese Ad-PD families, but was also present in eight controls (allele frequency, 1.7%). In addition, the variant was also found in three additional Ad-PD patients of the 89 Ad-PD index patients (allele frequency, 1.7%) and three of the 73 sPD patients (allele frequency, 2.2%). The previously reported nonsynonymous variant p.G2385R was identified in 2 of the 11 Japanese Ad-PD families (allele frequency, 9%), but not found in the 19 Caucasian families. Because recent studies in Taiwan have shown a higher frequency of p.G2385R in sPD than in controls (5.0 vs 2.5%, $P=0.012$),¹⁶ the frequencies of p.G2385R in controls, sPD and Ad-PD were further analyzed as described below.

Among the variants identified in Caucasian families, two (p.I1371V and p.G2019S) were initially missed in the analysis using GDAS software and subsequent visual inspection of the signals of undetermined

Table 1 Demographic characteristics of patients who participated in (a) comprehensive analysis of *LRRK2*; (b) frequency analysis of G2385R variant

	Japanese Ad-PD		Canadian Ad-PD	
(a)				
Number of probands	11		19	
Male	3 (27.3%)		9 (47.3%)	
Female	8 (72.7%)		10 (52.7%)	
Age at onset (years)	48.7 ± 9.8 (32–62)		51.6 ± 9.5 (26–70)	
Male	44.3 ± 11.6 (32–55)		52.6 ± 11.2 (37–70)	
Female	50.4 ± 9.3 (32–62)		50.6 ± 8.0 (26–70)	
	Ad-PD	sPD	Controls	
(b)				
Number of probands	89		73	
Male	33 (37.1%)		45 (61.6%)	
Female	56 (62.9%)		28 (38.4%)	
Age at onset (years)	53.5 ± 13.0 (25–76)		52.1 ± 14.8 (18–80)	
Male	51.5 ± 15.4 (25–67)		49.4 ± 15.3 (18–80)	
Female	54.0 ± 12.7 (34–76)		57.2 ± 12.4 (28–71)	

Abbreviations: Ad-PD, autosomal dominant PD; PD, Parkinson's disease; sPD, sporadic PD.

Table 2 Variations identified by comprehensive analysis of *LRRK2*

Exon	SNP ID ^a	Base change	Amino-acid change	Number of alleles (allele frequencies)	
				Japanese	Canadian
1	rs2256408	g to a	R50H	22 (100)	38 (100)
5	rs10878245	t to c	p.L153L	0 (0)	15 (39.5)
7	rs28365216	a to t	p.N238I	1 (4.5)	0 (0)
14	novel	g to a	p.A521A	0 (0)	1 (2.6)
	rs7308720	c to g	p.K551N	3 (13.6)	1 (2.6)
18	rs10878307	a to g	p.I723V	2 (9)	5 (13.2)
22	rs7966550	t to c	p.L953L	3 (13.6)	2 (5.3)
27	novel	c to g	p.N1221K	1 (4.5)	0 (0)
29	novel	g to t	p.R1320S	1 (4.5)	0 (0)
	rs17466213	a to g	p.I1371V	0 (0)	1 (2.6)
30	rs7133914	g to a	p.R1398H	3 (13.6)	0 (0)
	rs11175964	g to a	p.K1423K	3 (13.6)	1 (2.6)
32	rs35507033	g to a	p.R1514Q	0 (0)	1 (2.6)
34	rs1427263	a to g	p.G1624G	4 (18.2)	29 (76.3)
	rs11176013	t to c	p.K1637K	6 (27.3)	15 (39.5)
	rs11564148	t to a	p.S1647T	3 (13.6)	12 (31.5)
37	rs10878371	t to c	p.G1819G	10 (45.5)	14 (36.8)
41	rs34637584	g to a	p.G2019S	0 (0)	3 (7.9)
43	rs10878405	g to a	p.E2108E	4 (18.2)	10 (26.3)
48	rs34778348	g to a	p.G2385G	0 (0)	6 (15.8)
	rs33962975	g to a	p.G2385R	2 (9)	0 (0)
49	rs3761863	t to c	p.T2397M	6 (27.3)	14 (36.8)
IVS49–43	novel	t to c		2 (9)	2 (7.9)

^aSNP ID denotes single-nucleotide polymorphism identification obtained from dbSNP database.

base calls, but were independently identified by direct nucleotide sequence analyses. Retrospective analysis of the microarray signals of these two variants showed that the signals for p.I1371V could have been detected as a heterozygous variant, whereas the signals for p.G2019S would have been difficult to determine considering that one of the three patients with p.G2019S was called as having a homozygous wild-type sequence using GDAS software.

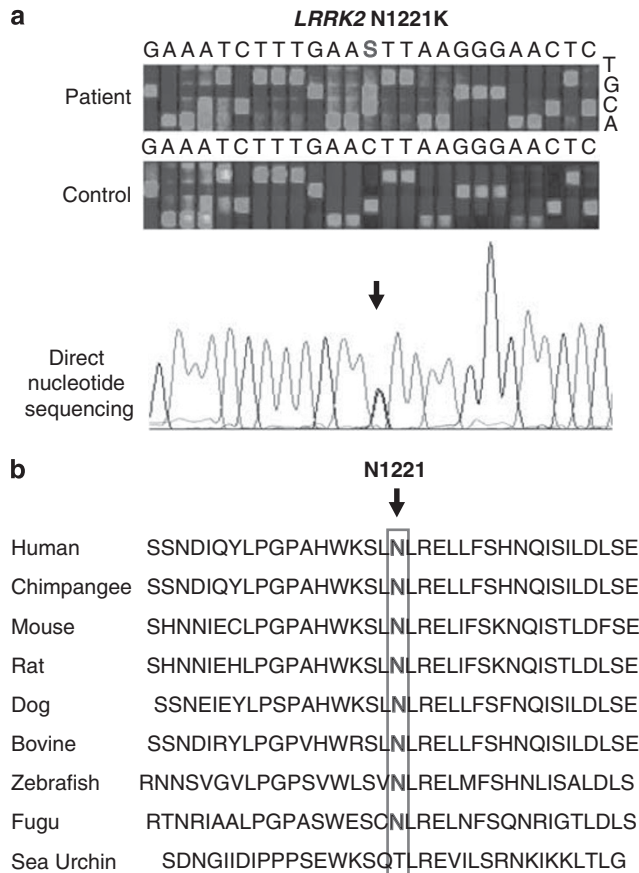


Figure 1 (a) Scan images of heterozygous *LRRK2* p.N1221K putative pathogenic mutation. Each column shows a base position, and each row shows a base call. In the center position, the base call was C in the control, whereas in the patients, the base calls were heterozygous C and G (upper panel). The mutation was confirmed by direct nucleotide sequencing. (b) Conservation of *LRRK2* amino-acid sequences among different animal species. The arginine residue at codon 1221 is highly conserved among species (shown in red). A full color version of this figure is available at the *Journal of Human Genetics* journal online.

Association of p.G2385R with Ad-PD

As mentioned above, we identified p.G2385R in two Japanese Ad-PD families. We first analyzed the frequency of p.G2385R in another data set of 89 Japanese Ad-PD families independent of the 11 Ad-PD families. We found that the seven index patients from the 89 Ad-PD families had p.G2385R. Among the seven Ad-PD families with p.G2385R, DNA samples were available from other pedigree members including affected patients in two families (families 2938 and 3045). In the two families (families 2955 and 2932), DNA samples from only other unaffected family members were available (Figure 2). p.G2385R did not show complete cosegregation with PD. Although cosegregation was suggested in family 3045, one affected patient did not carry p.G2385R in the family 2938. Furthermore, unaffected parents of the two other families (families 2955 and 2932) also carried the p.G2385R variant in a heterozygous state. Nevertheless, the frequency in 18 index patients of the 100 Ad-PD families of p.G2385R was significantly higher than that in controls (allele frequency, 9.0 vs 2.1%) ($\chi^2=16.32$, $P=5.34 \times 10^{-5}$) (Table 3). p.G2385R has recently been reported to be a polymorphism present in the Japanese control population, but overrepresented in sPD patients compared with controls (6.0 vs 2.4%, $P=1.24 \times 10^{-4}$).¹⁷ Given this report, we then analyzed the frequencies of p.G2385R in 73 sPD patients and 233 controls (Table 3). The frequency of the p.G2385R variant in sPD also

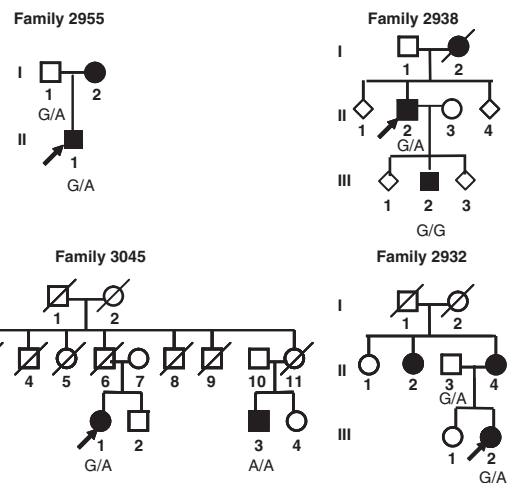


Figure 2 Pedigree charts in which cosegregation of *LRRK2* p.G2385R was examined. Affected individuals are indicated with black symbols. The proband is indicated with an arrow. Unaffected individuals are indicated with open symbols. Slashed symbols indicate deceased subjects. Genotype of each member available for cosegregation analysis is shown at each position. G: wild-type allele, A: p.G2385R allele (rs34778348:G>A).

Table 3 Genotype and allele frequencies of p.G2385R variant in Ad-PD, sPD and controls

	Number of cases	Genotype (%)			Allele (%)		χ^2 (P-value)	
		G/G	G/A	A/A	G	A	vs Control	vs sPD
Ad-PD	100	82 (82.0)	18 (18.0)	0 (0)	182 (91.0)	18 (9.0)	16.32 (5.34×10 ⁻⁵)*	4.22 (3.97×10 ⁻³)*
sPD	73	67 (90.5)	5 (6.8)	0(0)	141 (96.6)	5 (3.4)	0.76 (0.38)	
Control	233	223 (95.7)	10 (4.3)	0 (0)	456 (97.9)	10 (2.1)		

Abbreviations: Ad-PD, autosomal dominant PD; PD, Parkinson's disease; sPD, sporadic PD.
*These values reached statistical significance.

tended to be higher than that in controls (allele frequency, 3.4 vs 2.1%), although the difference did not reach statistical significance ($\chi^2=0.76$, $P=0.38$) (Table 3). The mean age at onset of Ad-PD in patients carrying p.G2385R was 55.3 ± 6.1 (18–67), which was not significantly different from that in patients not carrying the variant (51.8 ± 13.0 , $P=0.49$). The mean age at onset of sPD in patients carrying the p.G2385R variant was 60.2 ± 6.9 (52–71) years, which was similarly not significantly different from that of sPD in patients not carrying the variant (51.3 ± 15.0 , $P=0.19$). The two Ad-PD and five sPD patients with p.G2385R whose detailed clinical information was available showed a good response to levodopa therapy and no atypical features.

DISCUSSION

This study demonstrated that the spectrum and frequency of *LRRK2* variants vary with ethnic background. In Caucasians, p.G2019S is the most frequent in both Ad-PD and sPD, accounting for ~5% of Ad-PD patients and 1.6% of sPD patients.^{8,9,18} Many of the patients from European populations share a common haplotype, suggesting that they have a common founder originating in the Near East at least 4000 years ago.^{19,20} In contrast, no single mutation has been reported as being predominant in Asian populations. Although three Japanese index patients with p.G2019S were reported,^{21,22} we did not detect p.G2019S in our Japanese Ad-PD cohort, consistent with previous reports, showing that p.G2019S is rare in Asian cohorts.^{10,11,18,23} Indeed, the substantial differences in allele frequencies of the synonymous *LRRK2* polymorphisms (rs10878245, rs1427263, rs11176013, rs17466213 and rs3761863) highlight the genetic diversity between the Japanese and Caucasian Ad-PD patients (Table 2). These findings raise the possibility that mutational analysis focusing on previously identified mutation hotspots may underestimate the incidence of mutations; therefore, a comprehensive analysis of every coding exon is warranted.

Our comprehensive analysis of *LRRK2* revealed 22 variants in 30 Ad-PD families, supporting the usefulness of the DNA microarray-based resequencing system. However, it should also be noted that data analysis using GDAS software and subsequent visual inspection of the signals of undetermined base calls may miss variants, as experienced in the cases of p.I1371V and p.G2019S. Improvement of software and careful inspection of signals are important for accurate mutational detection.

In the Japanese Ad-PD families, we identified a novel nonsynonymous variation, p.N1221K. Although cosegregation could not be confirmed, it is possibly a pathogenic mutation for the following reasons: (1) p.N1221K was not found in 466 control chromosomes, (2) the amino-acid residue N1221 was highly conserved among different animal species and (3) it is located in the LRR domain, where some likely pathogenic mutations are clustered.^{24–26} The LRR domain resides in many proteins with diverse functions and provides a structural framework for the formation of protein–protein interactions.²⁷ The substitution of a charged amino-acid lysine for a neutral asparagine may disrupt protein–protein interactions, resulting in the dysfunction of the *LRRK2* protein. Identification of additional pedigrees harboring the mutation, cosegregation analysis and functional studies would be necessary to confirm the pathogenicity of the mutation.

The age at onset of the patient with p.N1221K was earlier than those of patients with previously reported mutations of *LRRK2* (the mean age at onset was in the range of 57–59 years).^{7,9,25} Considering that p.G2019S is the most frequent among the known mutations, the overall clinical presentation of patients with *LRRK2* mutations may

largely reflect that of patients with p.G2019S. Interestingly, a previously reported case of a patient with the p.S1228T mutation, located in the same exon and domain as in p.N1221K, showed a similarly early age at onset to the patient with p.N1221K, who developed symptoms at the age of 49.²⁵ It will be important to accumulate further detailed clinical information on patients with *LRRK2* mutations to evaluate the genotype–phenotype correlations.

In the Caucasian Ad-PD families, we identified two nonsynonymous variants, p. I1371V and p. R1514Q, both of which were initially reported as a putative pathogenic mutation.^{28,29} However, subsequent studies have revealed that these variants were identified in controls or not cosegregated within Ad-PD families, implying that they are not associated with an increased risk of the disease.^{30–32} It is often difficult to interpret the pathogenicity of each rare nonsynonymous variant in *LRRK2* because this gene is highly polymorphic and numerous nonsynonymous variants have been identified with diverse frequencies among different ethnic backgrounds. In this study, we could not conclude that the two nonsynonymous variants were pathogenic because large families appropriate for cosegregation analysis were not obtained and control data were not available, and further studies are required to evaluate the relevance of the variants to the pathogenesis of PD.

Of note, a known nonsynonymous variant R50H was found homozygously in all the pedigrees with the allele frequency of 100% in this study. Although the variant was initially registered as a nonsynonymous single-nucleotide polymorphism, a subsequent study on the comprehensive sequencing of *LRRK2* concluded that this variant should be a mistake in the consensus human genome sequence because it was present in all the cases and controls in their study.³³ The present study further supports the above conclusion that the consensus amino acid at position 50 in *LRRK2* protein is histidine instead of arginine. Future studies on the mutational analysis for *LRRK2* should take these results into account.

Heterozygosity for the p.G2385R variant has recently been demonstrated to be significantly more frequent in PD patients than in controls in Asian populations.^{16,17,34–39} One of these studies included 26 patients with a familial history of PD, among which six patients were p.G2385R carriers.⁴⁰ A meta-analysis on the basis of these studies involving 2205 PD patients and 1817 controls demonstrated average carrier rates of 9.3% in PD and 4% in controls.⁴¹ In this study, the frequency of the p.G2385R variant did not reach statistical significance between sPD patients and controls, probably due to relatively small sample size.

Intriguingly, we observed a significant overrepresentation of the p.G2385R variant in Ad-PD index patients compared with that in controls, although no complete cosegregation was observed. Note that the frequency of the p.G2385R variant in our control cohort was the same as that in a previous report (2.4%).¹⁷ In addition, the frequency of the p.G2385R variant also tended to be higher in Ad-PD patients than in sPD patients in accordance with a previous study.⁴⁰ Taken together, this study reinforced the notion that p.G2385R is a potential risk factor for PD. However, lack of evidences on the cosegregation suggested that other genetic factors should also be involved in the disease pathogenesis in the pedigrees with p.G2385R mutation. Analyses of p.G2385R in combination with recently identified genetic risk factors for PD such as *SNCA*^{42–44} and *GBA*^{45,46} would be important for evaluating the combined effects of these factors as well as for elucidating the molecular pathophysiology of PD.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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