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Short Report

Phase analysis identifies compound heterozygous deletions of the *PARK2* gene in patients with early-onset Parkinson disease

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Exon rearrangements and point mutations are common in *PARK2*, the most important causative gene of autosomal recessive early-onset Parkinson disease (EOPD). However, gene dosage analysis alone cannot conclusively determine the phase of exon rearrangements and the incidence of molecularly confirmed parkin-type EOPD may be underestimated. To fully characterize the mutation spectrum, we performed sequencing and gene dosage analyses of *SNCA*, *PARK2*, *PINK1*, and *PARK7* in 114 unrelated EOPD patients with onset age ≤40 years. Mutational phase of exon rearrangements was determined by reverse-transcriptase PCR (RT-PCR) and sequence analysis using a patient's own RNA. Fourteen different *PARK2* mutations (3 point mutations plus 11 exon rearrangements) were identified in 18 patients, comprising 1 homozygote (0.9%), 13 compound heterozygotes (11.4%), 3 single heterozygotes (2.6%), and 1 with unknown phase (0.9%). By phase determination, more than 80% (5 of 6) of patients with apparently contiguous multi-exon deletions and 30% (5 of 18) of all *PARK2* mutation carriers were additionally diagnosed as compound heterozygotes, respectively. This study shows that compound heterozygous mutations constituted a significant portion of patients with apparently contiguous multi-exon deletions. Phase determination is a prerequisite to molecular diagnosis for autosomal recessive EOPD, especially in subjects with *PARK2* exon rearrangements.

Conflict of interest

The authors declare no conflict of interest.

Key words: early-onset Parkinson disease – Korean – mutation – *PARK2* – phase determination

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Sporadic Parkinson disease (PD) is a major type of Parkinsonism. In recent years, familial forms have been described in a subset of PD, especially early-onset PD (EOPD) (1). At least nine monogenic causes constitute approximately 5–10% of PD: *SNCA*, *PARK2*, *UCHL1*, *PINK1*, *PARK7*, *LRRK2*, *ATP13A2*, *GIGYF2* and *HTRA2* (2). Among them, *PARK2* is the most important genetic cause of EOPD with autosomal recessive inheritance (1). Molecular diagnosis of a genetic disorder with autosomal recessive inheritance requires determination of the mutational phase, in cases that are not homozygous for a deleterious mutation. It is also necessary to establish genotype–phenotype relationship.

Exon rearrangement involving one or more exons accounts for 50–60% of all *PARK2* mutations (3, 4) and can be easily detected by gene dosage analysis such as multiplex ligation-dependent probe amplification (MLPA) (5). However, quantitative

analysis alone cannot conclusively determine the phase of exon rearrangements and the true incidence of molecularly confirmed parkin-type EOPD may be underestimated. So far, a few previous studies have analyzed the mutational phase of exon rearrangements (6, 7).

In this study, we aimed to characterize the mutation spectrum of *SNCA*, *PARK2*, *PINK1*, and *PARK7* among 114 Korean EOPD patients with a symptomatic onset age before or equal to 40 years. We performed sequencing and gene dosage analyses of these genes and analyzed the mutational phase of exon rearrangements.

Materials and methods

Subjects

This study enrolled 114 unrelated Korean EOPD patients with onset age ≤40 years (median age, 36; range, 12–40). They were clinically diagnosed with PD based on the UK Parkinson's Disease Society Brain Bank clinical diagnostic criteria (8). No consanguineous marriages occurred within any of the participants' individual families. Normal frequency of a novel sequence variant was determined using 171 healthy subjects (median age, 63; range, 37–83). The study was approved by the institutional review board. All subjects gave their consent for participation in this study.

Genomic DNA was extracted from peripheral blood by the PureGene DNA isolation kit (Gentra Systems, Minneapolis, MN). Total RNA was isolated from the lymphoblastoid cells of each patient using the RNeasy mini kit (Qiagen, Hilden, Germany).

Mutation analyses

Target genes

Four genes were analyzed: *SNCA* (NM_000345.2), *PARK2* (NM_004562.1), *PINK1* (NM_032409.2), and *PARK7* (NM_007262.4).

Sequence analyses

Target DNA was amplified by PCR and directly sequenced for detection of sequence variants in all coding exons and the flanking intronic regions of each gene (primers are available on request). Amplified products were purified by ExoSAP-IT treatment (USB, Cleveland, OH) and sequenced by the ABI PRISM 3730xl Genetic Analyzer (Applied Biosystems, Foster City, CA) using a BigDye Terminator Cycle Sequencing Ready Reaction Kit (Applied Biosystems). Obtained sequences were analyzed using Sequencher software version 4.6 (Gene Codes Corporation, Ann Arbor, MI).

Table 1. Mutations and UVs identified in this study

Gene	Sequence variant[a]	Number of alleles	Reference of previous report
PARK2	Deletion, exon 2; c.8-?_171+?del	4	(15)
	Deletion, exon 2–3; c.8-?_412+?del	1	(15)
	Deletion, exon 2–4; c.8-?_534+?del	2[b]	(15)
	Deletion, exon 3; c.172-?_412+?del	4	(15)
	Deletion, exon 3–4; c.172-?_534+?del	5	(15)
	Deletion, exon 4; c.413-?_534+?del	7	(15)
	Deletion, exon 5; c.535-?_618+?del	2	(15)
	Deletion, exon 6; c.619-?_734+?del	1	(15)
	Duplication, exon 2; c.8-?_171+?dup	1	(17)
	Duplication, exon 2–4; c.8-?_534+?dup	1	(15)
	Duplication, exon 6; c.619-?_734+?dup	1	(15)
	c.850G>C, p.Gly284Arg	1	(17)
	c.597dup, p.His200ThrfsX6	1	Novel frameshift mutation
	c.814C>A, p.Leu272Ile	2	UV (18, 19)
	c.1192G>A, p.Ala398Thr	1	(17)
PINK1	c.835C>T, p.Arg279Cys	1	Novel UV

MLPA, multiplex ligation-dependent probe amplification; UV, Unclassified variant.
[a]All genomic deletions and duplications were detected by MLPA.
[b]One patient had a single copy of exon 2–4 in *PARK2*; however, RNA specimen was unavailable for this patient.

Significance assessment for novel missense variants

The significance of each novel missense variant was assessed by (i) its allelic frequency in 171 normal control subjects, (ii) interspecies amino acid conservation of the mutated amino acid, (iii) in silico prediction for the effect of novel variants, (iv) the protein domain in which the mutation is located, and (v) familial segregation. The normal frequency of each variant was determined using PCR and denaturing high-performance liquid chromatography or restriction fragment length polymorphism analysis (primers are available on request). In silico prediction was performed by POLYPHEN (http://genetics.bwh.

Table 2. Mutational phase analysis for *PARK2* gene

	Gene dosage analysis[a]		Mutational phase	
Case	Exon involvement	Exon copy	Allele 1	Allele 2
1	Contiguous	One copy of exon 2–4	Del, exon 2	Del, exon 3–4
2	Contiguous	One copy of exon 3–4	Del, exon 3	Del, exon 4
3	Contiguous	One copy of exon 3–4	Del, exon 3	Del, exon 4
4	Contiguous	One copy of exon 3–4	Del, exon 3	Del, exon 4
5	Contiguous	One copy of exon 3–4	Del, exon 3–4	Normal
6	Contiguous	One copy of exon 2–4	Del, exon 2–4	c.850G>C (p.Gly284Arg)
7	Contiguous	One copy of exon 2–4	Del, exon 2–4 (phase unknown)	
8	Discontiguous	One copy of exon 3–4; three copies of exon 6	Del, exon 3–4	Dup, exon 6
9	Discontiguous	One copy of exon 2–3; one copy of exon 5	Del, exon 2–3	Del, exon 5
10	Discontiguous	One copy of exon 4; one copy of exon 6	Del, exon 4	Del, exon 6
11	Discontiguous	One copy of exon 2; one copy of exon 4	Del, exon 2	Del, exon 4
12	Discontiguous	One copy of exon 5; three copies of exon 2–4	Dup, exon 2–4	Del, exon 5
13	Exon overlap	One copy of exon 3; zero copy of exon 4	Del, exon 3–4	Del, exon 4
14	Exon overlap	Zero copy of exon 3; one copy of exon 4	Del, exon 3–4	Del, exon 3
15	Exon overlap	Zero copy of exon 2	Del, exon 2	Del, exon 2
16	Single exon	One copy of exon 4	Del, exon 4	c.597dup (p.His200ThrfsX6)
17	Single exon	Three copies of exon 2	Dup, exon 2	Normal
18	No rearrangement	No deletion/duplication	c.1192G>A (p.Ala398Thr)	Normal

Del, deletion; Dup, duplication; MLPA, multiplex ligation-dependent probe amplification.
[a]All genomic deletions and duplications were detected by MLPA.

harvard.edu/pph/) and SIFT (http://blocks.fhcrc.org/sift/SIFT.html) (9, 10).

Gene dosage analyses

Exon rearrangements were determined by MLPA, P051/P052 kit (MRC-Holland, Amsterdam, The Netherlands). DNA denaturation, probe-target sequence hybridization, ligation, and multiplex PCR were performed according to the manufacturer's protocol. Amplified products were separated using the ABI PRISM 3130xl Genetic Analyzer (Applied Biosystems) and analyzed by GeneMarker software version 1.51 (SoftGenetics LLC, State College, PA). For the detected dosage alteration in MLPA probes, the absence of sequence variations located within probes near ligation sites was confirmed in each subject.

Reverse-transcriptase PCR (RT-PCR) and sequence analysis for phase determination

For patients carrying exon rearrangements, phase was determined by RT-PCR and subsequent sequencing. Reverse transcription was performed with 1 μg of RNA using Expand reverse transcriptase (Roche Diagnostics, Mannheim, Germany) and downstream-specific primer RNA-11R (Table S1, supporting information). Two rounds of semi-nested PCR were performed and the compound heterozygote was diagnosed by the presence of bands with the expected sizes of exon rearrangements in the MLPA result. Sequence analysis of the RT-PCR product reconfirmed the sequence of the deleted or duplicated exon margin.

Results

Mutations

Sequence analysis revealed three different *PARK2* mutations in three patients (Table 1). Two mutations, c.850G>C (p.Gly284Arg) and c.1192G>A (p.Ala398Thr), were previously reported as causes of EOPD, and the other one, c.597dup (p.His200 ThrfsX6), was previously unreported. The *PARK2*: c.597dup induces a frameshift and premature termination of the protein parkin; therefore, it was classified as a pathogenic mutation.

Gene dosage analysis revealed 11 different *PARK2* exon rearrangements in 17 patients (Table 1). In three patients, *PARK2* rearrangements involved single exon (Table 2). In other patients, rearrangements involved multiple exons and it was unclear whether they were contiguous.

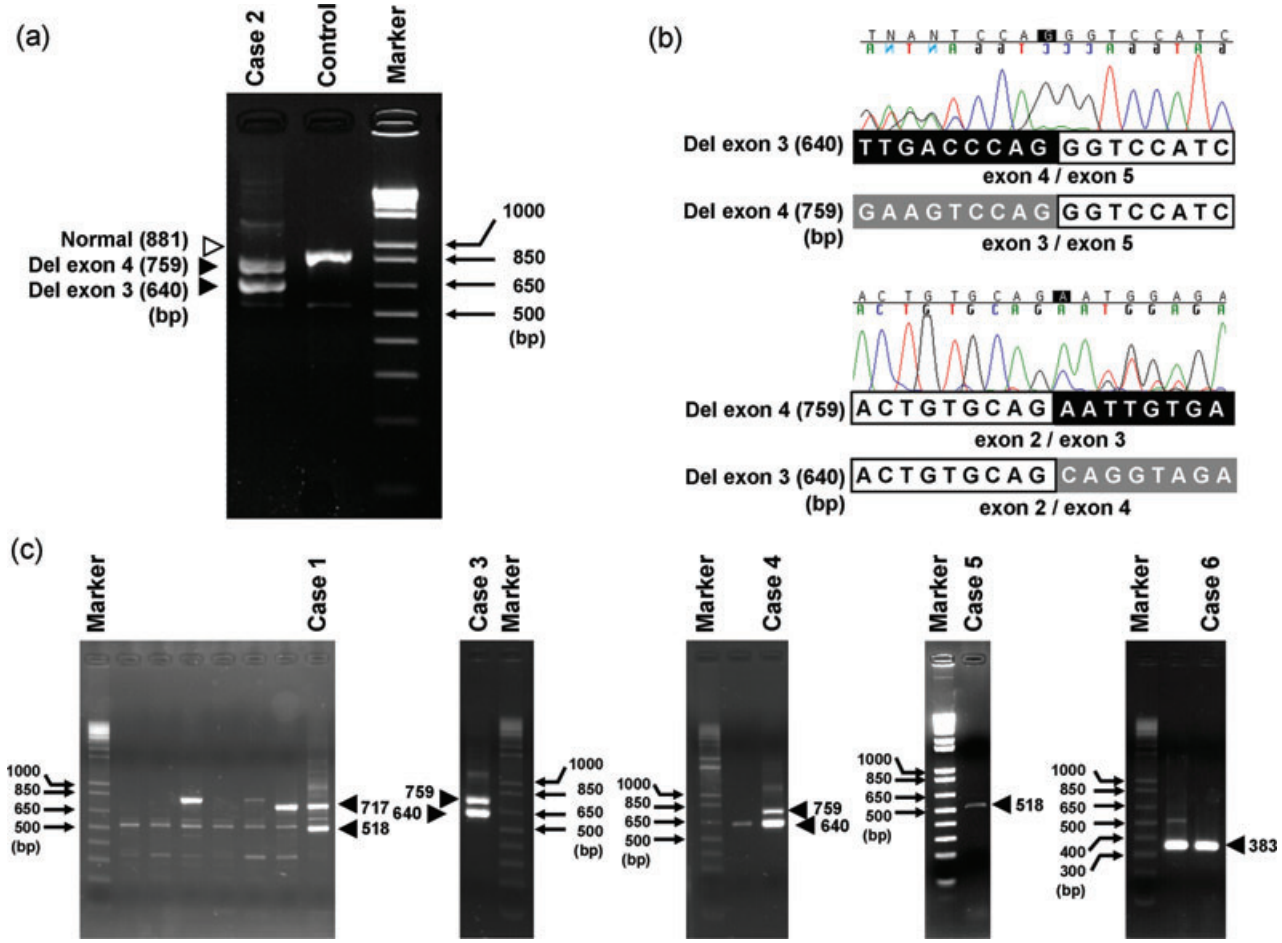

Fig. 1. Results of RT-PCR and sequence analysis to determine the mutational phase of exon rearrangements. (**a**) For a patient (case 2) with exon 3–4 deletions on multiplex ligation-dependent probe amplification, RT-PCR revealed two separate shortened bands suggesting two different deletions, and (**b**) subsequent sequencing confirmed that two bands are produced by two single deletions of exons 3 and 4, respectively. (**c**) Through these mutational phase analyses, four patients harboring an apparent contiguous multi-exon deletion were also determined as compound heterozygotes: case 1, exon 2 deletion and exon 3–4 deletion; case 3, exon 3 deletion and exon 4 deletion; case 4, exon 3 deletion and exon 4 deletion; case 6, exon 2–4 deletion and c.850G>C. One patient was single heterozygote for *PARK2* exon rearrangement: case 5, exon 3–4 deletion. The size of each exon-deleted RT-PCR product is as follows: normal transcript, 881 bp; exon 2 deletion, 717 bp; exon 3 deletion, 640 bp; exon 4 deletion, 759 bp; exon 3–4 deletion, 518 bp; exon 2–4 deletion, 383 bp using primer RNA-1F and RNA-8R (Table S1, supporting information).

Seven of the 17 patients harbored a contiguous multi-exon deletion and we could not determine their mutational phase based on gene dosage results alone. We analyzed the mutational phase of six patients whose RNA was available using RT-PCR and subsequent sequencing (Fig. 1). Among them, five patients were compound heterozygous for adjacent exon deletions and only one was single heterozygous for *PARK2* exon rearrangement. No pathogenic point mutation or exon rearrangement was identified in the *SNCA*, *PINK1*, and *PARK7* genes.

Finally, we identified 18 EOPD patients with *PARK2* mutations, comprising of one homozygote (0.9%), 13 compound heterozygotes (11.4%), three single heterozygotes (2.6%), and one with unknown phase (0.9%) (Table 2). Patients confirmed with autosomal recessive EOPD were 12.3% of all 114 patients participating in the study and 77.8% of *PARK2* mutation carriers.

Unclassified variants (UVs) and polymorphism

Two novel missense variants were identified in this study, including *PINK1* : c.835C>T (p.Arg279Cys) and *SNCA*: c.158C>T (p.Ala53Val) (Table 3 and Fig. S1, supporting information). The *PINK1* variant (c.835C>T) was located in the protein kinase domain and the amino acid was highly conserved across species. However, it was classified as a sequence variant of unknown significance because it was also identified in normal controls (EOPD *vs* normal controls, 0.43 *vs* 0.58%) and was in a single heterozygous state.

Table 3. Significance assessment of unclassified missense variants identified in this study

Sequence variant	*PINK1*:c.835C>T (p.Arg279Cys)	*SNCA*:c.158C>T (p.Ala53Val)	*PARK2*:c.814C>A (p.Leu272Ile)
Mutation at the same codon	p.Arg279His (20)	p.Ala53Thr (21)	None
Protein domain	Protein kinase	N-terminal region	RING1
POLYPHEN prediction	Probably damaging	Benign	Benign
SIFT prediction	Tolerated	Affect protein function	Affect protein function
Amino acid conservation	Highly conserved	Poorly conserved	Highly conserved
Normal allele frequency	2/342 (0.58%)	1/342 (0.29%)	0/342 (0%)
Familial segregation	Not tested	No segregation[a]	Not tested

[a]His 81-year-old mother and three siblings (62, 57, and 50 years old, respectively) were heterozygous for this variant, otherwise asymptomatic and neurologically normal.

The *SNCA* variant (c.158C>T) was identified in healthy family members as well as normal controls, and the amino acid was poorly conserved. Therefore, it was classified as a rare polymorphism in the causative gene for autosomal dominant EOPD.

The *PARK2*: c.814C>A (p.Leu272Ile) has been previously reported and was also classified as a UV in this study because the amino acid at this codon was highly conserved and the sequence variant was not identified in our normal controls.

Discussion

This study suggests that a significant portion of EOPD patients with apparent contiguous multi-exon deletions may actually be compound heterozygous for two different adjacent exon deletions. By phase determination, more than 80% (5 of 6) of patients with contiguous multi-exon deletions and 30% (5 of 18) of all *PARK2* mutation carriers were diagnosed as compound heterozygotes, respectively. Therefore, quite a number of contiguous multi-exon deletions or duplications that were reported in previous studies and were unknown for phase are likely to be compound heterozygous ones. This study also indicates that the RT-PCR alone can determine the mutational phase of many patients with contiguous multi-exon deletions, even when familial segregation analysis is not available for them.

Undetermined mutational phase could lead to a false conclusion on the contribution of single heterozygous mutations to the development or clinical severity of EOPD (6, 11, 12). In our study, considering contiguous exon rearrangements as single heterozygotes, onset age was statistically different between single heterozygotes and patients without mutation. However, this difference between the two patient groups was no longer observed after determination of the mutational phase.

The mutation spectrum revealed in this study was different from those in other studies in Korean population (13, 14). The proportion of molecularly confirmed cases with two pathogenic mutant alleles was very high (4.2–5.5 *vs* 12.3%). One study reported that 5.5% (3/55) of patients had two *PARK2* mutations (13). The other reported that 4.2% (3/72) of patients had two mutations and 12.5% (9/72) of patients had one or two mutations (14). In this study, 15.8% (18/114) were mutation-carrying patients and 12.3% (14/114) were molecularly confirmed cases with two *PARK2* mutant alleles. Our higher mutation identification rate is probably based on the followings: we analyzed larger number of EOPD patients and they were more strictly selected according to an earlier age of onset (≤40 years) than those in previous studies.

The *PARK2* gene was the most predominant genetic cause and exon rearrangement was far more common than point mutation in this population. We identified 20 alleles with single exon rearrangement and nine alleles with multi-exon rearrangement. Exon rearrangements were clustered between exons 2 and 6, and single deletions of exon 3 or 4 and contiguous exon 3–4 deletions were most common in this population (Table 1). These locations of frequent exon rearrangements in Koreans were similar to those in previous reports of other ethnicities (15, 16). Allele frequency of exon rearrangements was about ten times higher than that of point mutations in all mutation carriers (0.91 *vs* 0.09). This high contribution of exon rearrangements has to be considered in the molecular diagnosis of EOPD in this population.

In conclusion, this study shows that phase determination is prerequisite to molecular diagnosis for autosomal recessive EOPD, especially in subjects with *PARK2* exon rearrangements.

Supporting Information

The following Supporting information is available for this article:

Fig. S1. Three unclassified variants identified in this study: *PINK1*:c.835C>T (p.Arg279Cys), *SNCA*:c.158C>T (p.Ala53Val),

and *PARK2*:c.814C>A (p.Leu272Ile). (**a**) Electropherograms of these sequence variants (arrow): the upper panel shows the sequence of the normal control and the lower panel shows the sequence of the patient. (**b**) Amino acid conservation across species: arginine at codon 279 in the *PINK1* and leucine at codon 272 in the *PARK2* are highly conserved across most species, but alanine at codon 53 in *SNCA* is poorly conserved.

Table S1. Primers and PCR conditions for semi-nested RT-PCR of the *PARK2* gene.

Additional Supporting information may be found in the online version of this article.

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