

Autosomal Recessive Juvenile Parkinsonism (AR-JP): Genetic Diagnosis

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1. Introduction

Autosomal recessive juvenile parkinsonism (AR-JP) is a familial levodopa-responsive parkinsonism resulting from Lewy body negative degeneration of nigral neurons in the zona compacta of the substantia nigra (1–4). The first proposal for a distinct clinical entity with recessively inherited parkinsonism was made in Japan and was termed “paralysis agitans with marked diurnal fluctuations of symptoms” (1). This syndrome was later designated as autosomal recessive form of juvenile parkinsonism (2). It was subsequently found to be linked to the 17-cM region on chromosome 6q25.2-27, and the locus was recently designated Park2 (3,5). Through the study of a patient who had homozygous microdeletion of the marker D6S305 (5), the responsible gene was identified by positional cloning and was designated *parkin* (6). Linkage and mutation analysis to date have shown that founders of mutations in this gene are multiple and widely distributed in the world (7–13). Abnormalities in this gene, which are specific for AR-JP, include homozygous exonic deletions, small deletions, and point mutations. The presence of homozygous exonic deletions strengthens the notion that nigral neurodegeneration in AR-JP is caused by loss of function of the parkin protein.

1.1. Assessment of the AR-JP Phenotype

1.1.1. Clinical and Pathologic Manifestations of AR-JP

The cardinal features of AR-JP are early-onset parkinsonism with a benign course and remarkable response to levodopa. The following clinical features are also important to support the diagnosis of AR-JP (Table 1):

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Table 1
Major and Minor Manifestations Useful for the Clinicopathologic Diagnosis of AR-JP

| Type of finding | Feature |
|-------------------------|--|
| Major clinical features | <p>Early-onset parkinsonism (mean age 27.0 ± 9.0 years; range: 8–58 yr)</p> <p>A clear levodopa-response</p> <p>Frequent and early dopa induced dyskinesias and wearing-off phenomenon</p> <p>No dementia and rare autonomic dysfunction</p> <p>Extremely slow progression (Hoehn-Yahr stage 2.6 ± 0.7, after 20–30 yr from onset)</p> |
| Minor clinical features | <p>Sleep benefit (improvement of symptoms after sleep lasting 30–120 min)</p> <p>Mild foot dystonia (dorsiflexion of big toe or pes equinovarus)</p> <p>Fine postural tremor</p> <p>Hyperreflexia with negative Babinski sign</p> |
| Pathological findings | <p>Lewy body-negative neuron loss with severe gliosis in the substantia nigra pars compacta</p> <p>Mild neuron loss in the locus ceruleus</p> |

Data from **ref. 4**.

1. Mild focal dystonia, which often manifests as unilateral foot dystonia-dorsiflexion of the big toe or pes equinovarus deformity. Dorsiflexion of the big toe can be easily observed when the patient sits on a high chair or walks with bare feet. In some cases, truncal dystonia is the first symptom.
2. Sleep benefit, which can be identified by asking patients whether their parkinsonian symptoms improve after naps, or whether their symptoms are much milder upon awakening in the morning compared with the evening.
3. Extremely slow progression of the disease and absence of dementia even in the terminal stages of the disease.
4. Rare occurrence of autonomic dysfunction such as constipation or neurogenic bladder.
5. Fine postural finger tremor.
6. Hyperactive deep tendon reflexes with a negative Babinski sign.
7. Dopa-induced dyskinesia, which soon follows the dramatic dopa responsiveness.
8. Wearing-off phenomenon, which is frequently encountered in a relatively early phase of the disease.

1.1.2. Family Interview

Family interviews typically reveal multiple affected individuals in one generation with no appearance of the disease in previous generations or offspring

of patients. Thus, if the patient has no siblings, AR-JP can manifest as a sporadic early-onset parkinsonism. Although 51% of AR-JP families (9/17) have consanguineous marriages (4), a sufficiently large proportion (49%) have no history of consanguinity despite exhaustive family interviews (4). Nevertheless, patients from these non-consanguineous families frequently have homozygous haplotypes (63%; see **Subheading 1.2.4.**), which indicates the presence of an ancient consanguineous loop. In such cases, the parents' families frequently originated from the same geographic area.

1.2. Analysis of Mutations in the parkin Gene

1.2.1. Structure and Expression of the parkin Gene

The *parkin* gene consists of 12 exons encoding 465 amino acids, with a molecular weight of 51,652 D (**Fig. 1**). The full-length cDNA, which has been isolated from human skeletal muscle and fetal brain cDNA libraries consists of 2860 bp with an open reading frame of 1395 bp. The N-terminal 76 amino acid residues show homology to ubiquitin (65% positive, 33% identical). The characteristic cysteine-rich motif (Cys-X2-Cys-X9-Cys-X1-His-X2-Cys-X4—Cys-X4-Cys-X2-Cys) is also found at the C-terminus of parkin. The *parkin* gene is ubiquitously transcribed. Northern blot analysis using full-length *parkin* cDNA as probe revealed a 4.5 kb mRNA in almost all tissues (6). In the brain, *parkin* mRNA is present in several regions, including cerebellum, substantia nigra, cerebral cortex, brainstem, putamen, caudate, hippocampus, amygdala, and thalamus. Reverse transcriptase polymerase chain reaction (RT-PCR) analysis using leukocyte RNA revealed no full-length mRNA but a shorter transcript in which exons 3, 4, and 5 are spliced out. In the brain, the full-length transcript, as well as a small amount of mRNA with a spliced-out exon 5, has been detected by RT-PCR (14).

1.2.2. Analysis of Exon Deletions by Genomic PCR

A wide variety of deletion mutations in the *parkin* gene have been reported so far (**Table 2**). If the patient is homozygous for the deletion, it is detectable by lack of a genomic PCR product using intron primers encompassing the deleted exons. However, if a patient is heterozygous for the deletions (compound heterozygote: see **Note 1**), only the exon whose deletion is shared by both chromosomes fails to be amplified. If no part of the deletion is shared between the two chromosomes, exon PCR cannot detect any deletion. For example, if an individual receives exon 3 deletion from the father and exon 4 deletion from the mother, exon PCR cannot detect any deletion. Southern blot analysis is not dependable for evaluation of such small changes in gene dosage. Accordingly, when the patient shows a heterozygous haplotype for mark-

M[○]I[○]V[○]E[○]VR[○]FN[○]SSHG[○]FP[○]VE[○]VD[○]SD[○]TS[○]IF[○]Q[○]L[○]KE[○]V[○]VA[○]K[○]RO[○]GV[○]PA[○]D[○]Q[○]LR[○]VI[○]FA[○]G[○]K[○]EL[○]R[○]ND[○]W[○]
T[○]V[○]Q[○]CD[○]LD[○]Q[○]Q[○]SI[○]VH[○]IV[○]Q[○]RP[○]WR[○]K[○]G[○]Q[○]EM[○]NA[○]TG[○]GD[○]DP[○]RNA[○]AG[○]GC[○]ER[○]P[○]Q[○]SL[○]TR[○]VD[○]L[○]
S[○]SS[○]VL[○]PG[○]DS[○]VGL[○]AV[○]IL[○]HT[○]DS[○]SR[○]K[○]D[○]SP[○]AG[○]SP[○]AG[○]RS[○]I[○]Y[○]NS[○]FF[○]Y[○]Y[○]CK[○]GP[○]C[○]Q[○]R[○]V[○]Q[○]PG[○]K[○]
L[○]R[○]V[○]Q[○]C[○]ST[○]CR[○]Q[○]AT[○]L[○]T[○]L[○]T[○]Q[○]GP[○]SC[○]W[○]DD[○]V[○]L[○]IP[○]NR[○]MS[○]GE[○]C[○]Q[○]SP[○]HC[○]PG[○]TS[○]AE[○]FF[○]K[○]CG[○]AH[○]
P[○]TS[○]D[○]K[○]ET[○]P[○]VA[○]L[○]H[○]LI[○]AT[○]NS[○]R[○]NI[○]T[○]C[○]IT[○]CT[○]D[○]V[○]R[○]SP[○]V[○]L[○]VF[○]Q[○]C[○]NS[○]R[○]H[○]VI[○]CL[○]DC[○]FH[○]LY[○]CV[○]T[○]
R[○]L[○]ND[○]R[○]Q[○]FV[○]HD[○]P[○]Q[○]L[○]G[○]Y[○]SL[○]PC[○]V[○]AG[○]CP[○]NS[○]L[○]I[○]KE[○]L[○]H[○]FR[○]IL[○]G[○]EE[○]Q[○]Y[○]NR[○]Y[○]Q[○]Q[○]Y[○]GA[○]EE[○]CV[○]L[○]Q[○]
M[○]GG[○]V[○]LC[○]PR[○]PG[○]CG[○]AG[○]LL[○]PE[○]PD[○]Q[○]R[○]K[○]VT[○]CE[○]GG[○]N[○]GL[○]G[○]CG[○]AF[○]CR[○]EC[○]KE[○]AY[○]HE[○]GE[○]CS[○]AV[○]
F[○]E[○]AS[○]G[○]TT[○]T[○]Q[○]AY[○]R[○]VD[○]ER[○]AA[○]E[○]Q[○]AR[○]WE[○]AA[○]SK[○]ET[○]IK[○]KT[○]TK[○]PC[○]PR[○]CH[○]VP[○]VE[○]KN[○]GG[○]CM[○]H[○]
M[○]K[○]CP[○]Q[○]P[○]Q[○]CR[○]LE[○]WC[○]WN[○]CG[○]CE[○]WN[○]RV[○]CM[○]GD[○]HW[○]FD[○]V[○]*

Fig. 1. Exon boundaries in the parkin protein. Open circles, exon boundary breaks three nucleotide amino acid codes; closed circles, exon boundary does not break the amino acid code. Ubiquitin-like sequences in the N-terminal portion of parkin protein are underlined. The conserved site of polyubiquitination (Lys at 48) is shown by asterisks. A ring finger-like cysteine-rich motif at the C-terminal portion is indicated by underlined cysteine (C) and histidine (H) residues within this motif.

ers on the AR-JP locus, negative results from exon PCR do not necessarily mean that the patient has no deletion in the AR-JP gene.

To date, exon PCR has been effective for the detection of deletions in 57% of chromosome 6q-linked recessive juvenile parkinsonism (12 of 21 families) in Japan and in 25% (3 of 12 families) in Europe and North Africa (11). Thus, 25–57% of clinical AR-JP can be detected by exon PCR.

1.2.3. Exon Sequencing

When exon PCR shows no deletion, the next step is to sequence each exon and its boundaries. A wide variety of point mutations in the *parkin* gene have been reported so far (Table 2).

Homozygous one-point mutations, small insertions or deletions at the same nucleotide site on both chromosomes could be detected. Alternatively, if the patient is a compound heterozygote (see Note 1), one-point heterozygous mutation at the same nucleotide position might also be detected. When a heterozygous point mutation is observed, the presence of a compound heterozygote with a deletion in the other chromosome is possible. When two-point mutations are observed at different sites, it is necessary to exclude the possible presence of two mutations residing on the same chromosome. This can be done by sequencing a carrier who has only one disease chromosome, which can be detected by haplotype analysis. If only one of these two-point mutations is

Table 2
Mutations in the *parkin* Gene

| |
|---------------------------------------|
| Exonic deletions detected by exon PCR |
| Exon 3 |
| Exons 3, 4 |
| Exons 3, 4, 5, 6, 7 |
| Exon 4 |
| Exons 4, 5, 6 |
| Exon 5 |
| Exons 5, 6, 7 |
| Exons 8, 9 |
| Point mutations |
| Lys161Asn (exon 4) |
| Thr240Arg (exon 6) |
| Arg256Cys (exon 7) |
| Arg275Trp (exon 7) |
| Thr415Asn (exon 11) |
| Gln311Stop (exon 8) |
| Trp453Stop (exon 12) |
| Small deletions or insertions |
| 202-3del (exon 2) |
| 255del (exon 3) |
| 321-2ins (exon 3) |
| 535del (exon 5) |
| Polymorphic mutations |
| Ser167Asn (exon 4) |
| Arg366Trp (exon 10) |
| Val380Leu (exon 10) |
| Asp394Asn (exon 11) |

Data from refs. 9–12, 14, 15, and 19.

observed in the carrier, the patient is a compound heterozygote. If both of the two-point mutations are present in the carrier, the patient may have two point mutations in one chromosome. In the latter case, it is still possible that the patient is a compound heterozygote with a deletion in one chromosome and two-point mutations in the other. When a new homozygous one-point mutation is identified, the possibility of polymorphic mutation should be assessed. Several polymorphic mutations in the *parkin* gene have been reported (**Table 2**) (13,15).

1.2.4. Haplotype Analysis

As mentioned above, haplotype analysis is mandatory to interpret correctly the results of exon deletions and point mutations (see **Note 2**). If an affected

patient has a heterozygous haplotype for the *parkin* gene, he/she is expected to be a compound heterozygote, receiving different mutations from each parent. In AR-JP derived from a consanguineous marriage, the patient usually receives the identical mutation from both parents, and thus should be homozygous for polymorphic marker alleles located in and around the *parkin* gene. However, in AR-JP, mutations in the *parkin* gene are variable and widely distributed in the world. This multiple-founder effect increases the likelihood of the occurrence of the disease from nonconsanguineous marriages, resulting in compound heterozygotes.

It should be noted that although the normal carrier state (heterozygote) of a deletion cannot be detected by conventional exon PCR, it can be detected by haplotype analysis if the individual belongs to the same family as the affected proband and the parents have heterozygous haplotypes (**Figs. 2–4**). When only patients' samples are available, it is desirable to calculate allele frequencies of the markers in the general population from which affected families originate. If the frequencies of the marker alleles are rare, haplotype homozygosity alone is sufficient to indicate the true linkage of the haplotype to the disease (*see Note 3*).

1.2.5. Analysis of *parkin* mRNA and Protein

Absence or truncation of *parkin* transcripts can be detected by RT-PCR using tissue RNA samples. The presence of tissue-specific splicing of *parkin* transcripts should be taken into consideration. For example, full-length *parkin* transcript is absent in peripheral leukocytes (**14**). When a specific antibody is available, Western blot analysis using tissue samples can detect abnormalities of parkin translated products. Analysis of the *parkin* mRNA and protein has just begun, and further studies should become available in the near future. Such analyses would be helpful in the diagnosis of AR-JP when genomic studies are not informative.

1.2.6. Perspectives

Even when PCR-based studies of homozygous exonic deletions and point mutations are negative in a particular patient, the diagnosis of AR-JP cannot be excluded if haplotype analysis shows a heterozygous haplotype. Individual patients might be compound heterozygotes having two different exonic deletions that do not share a common segment. When a heterozygous point mutation is present in a patient, a compound state with one deletion and one point mutation should be evaluated. Thus, without a sensitive method to detect small changes in gene dosage such as heterozygous deletions, the diagnosis of AR-JP should depend on the efforts to put together the results of PCR-based analysis of the mutation and haplotype studies of the pedigree.

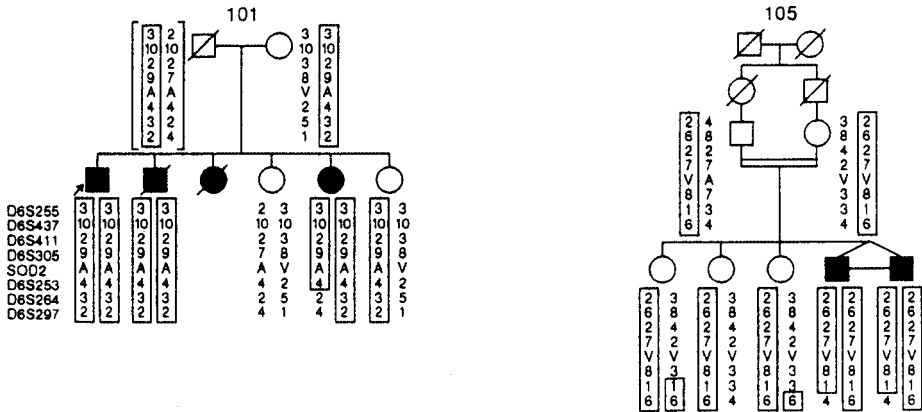


Fig. 2. Haplotype analysis and carrier detection in AR-JP pedigrees (3). Homozygous segregation of haplotypes and diagnosis of carrier state are possible in these two AR-JP families. The haplotype of the disease chromosome is enclosed by the rectangle. Markers used are D6S441, D6S255, D6S437, D6S305, alanine (A)/valine (V) dipolymorphism of MnSOD, D6S253, D6S264, and D6S297. As seen in pedigree 101, multiple affected siblings and homozygous segregation are frequently seen with no apparent consanguinity. In this family, the second daughter is not a carrier of the disease chromosome. However, both parents and the fourth daughter are carriers (heterozygote), with one disease chromosome whose haplotype is 3-10-2-9-A-4-3-2. All affected individuals are homozygous for the haplotype 3-10-2-9-A-4-3-2. Recombination is observed between markers D6S253 and D6S264 on the paternal chromosome of the third affected daughter. This family has exon 4 deletion. Note the first-degree cousin marriage in family 105. Only the patients (monozygotic twins) show homozygosity of the disease chromosome with the haplotype 2-6-2-7-V-8-1. All other siblings and their parents are carriers of the disease chromosome. Several recombinations are observed in members of this family except in the second unaffected daughter. This family has exon 5 deletion.

The existence of multiple founder mutations in the *parkin* gene and the high proportion of nonconsanguinity in AR-JP pedigrees (49%) indicate a high frequency of compound heterozygotes and asymptomatic carriers of *parkin* mutations in the normal population, resulting in a potentially high prevalence of sporadic cases of AR-JP (4). The major obstacle for assessing the latter possibility is the difficulty in detecting deletion heterozygotes.

Recently, real-time PCR monitoring by fluorescent-energy transfer techniques such as TaqMan or LightCycler system have been introduced to detect such small differences in gene dosage (16). These technical improvements could enable the detection of deletion heterozygotes in the *parkin* gene. At the

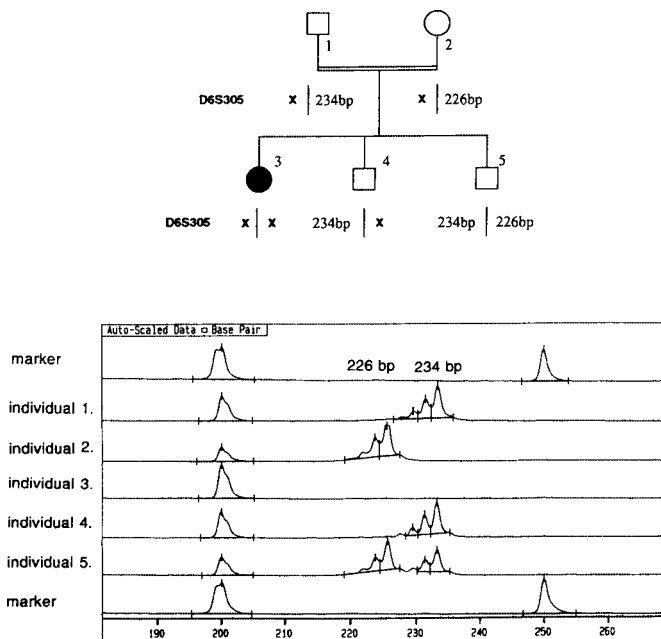


Fig. 3. Allotype analysis of the microsatellite marker D6S305 in the family of an AR-JP patient using the Pharmacia ALF2 Fragment manager (5). Data are obtained by the Pharmacia ALF2 sequencer and analyzed by Fragment manager software. FITC-labeled PCR products of the microsatellite marker D6S305 were electrophoresed. In each of these lanes, size markers (200 and 300 bp) were also run. This zoomed-in figure does not show the peak at 300 bp. In lanes designated as markers, a 50-bp size ladder was run. Note the 200- and 250-bp peaks in each marker lane. The ordinate represents nucleotide length (bp). PCR products generate a complex of peaks, with several smaller peaks are located left of the highest peak, which represents shorter products generated by the skipping phenomenon of amplification. This phenomenon is often observed in amplification of short nucleotide repeats. The length difference between each of these skipping peaks is 2 bp, because D6S305 is a dinucleotide repeat polymorphic marker. Two alleles (226 and 234 bp) are seen in this family. Individuals 1 and 2 are parents who are first cousins. Individual 1 shows a single allele (234 bp). Individual 2 shows a single allele, which is different in size from that of individual 1 (226 bp). If individuals 1 and 2 are homozygotes, all offspring should show a heterozygous allotype (226/234 bp). However, individual 4 shows a single allele (234 bp), indicating that this person received a null (deleted) allele (shown as X in the family pedigree) from individual 2. This in turn suggests that individual 2 has heterozygous deletion of this marker (X/226 bp). On the other hand, individual 3, who has clinical AR-JP, shows no PCR product, indicating that she received two deleted alleles, one from each parent (X/X). The latter observation means that individual 1 also has a heterozygous deletion of the marker (X/234 bp). Individual 5 shows a heterozygous allotype (226/234 bp), indicating that he received no deleted allele from either parent. These findings taken together indicate that the responsible gene for AR-JP resides in close proximity to D6S305.

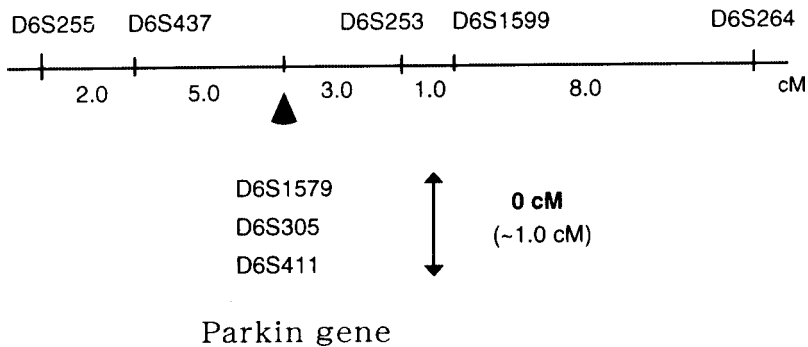


Fig. 4. Genetic map of polymorphic microsatellite markers in and around the *parkin* gene on chromosome 6q25.2-27 (3-5). The microsatellite marker D6S305 is located within the *parkin* gene (5,6).

same time, the full sequencing of the genomic region in and around the *parkin* gene is in progress, which will enable the detection of mutations in the noncoding region of this gene as well.

α -Synuclein aggregation is considered a major cause of Lewy body formation. Nonetheless, the cell death pathway triggered by α -synuclein aggregation is not clear. The unique feature of neuronal death in AR-JP, namely, absence of Lewy body formation, suggests the possibility that the downstream event in the cell death cascade triggered by α -synuclein aggregation might share the same biochemical pathway involving *parkin* (17). Genetic analysis of mutations in the *parkin* gene in AR-JP patients will eventually contribute to the elucidation of the functional role of parkin in the pathogenesis of Parkinson's disease.

2. MATERIALS

2.1. Exon Deletions

1. Chimeric primers with M13 universal and reverse primer sequences at their 5' ends are used for exon PCR as well as for exon sequencing (Table 3, see Note 4).
2. Ampli Taq Gold DNA polymerase (Perkin-Elmer, Applied Biosystems Division, Foster City, CA).
3. 10X PCR buffer: 500 mM KCl, 100 mM MgCl₂, 0.1% gelatin.
4. 10 mM dNTPs.
5. PCR thermal cycler.

2.2. Exon Sequencing

1. The PCR product obtained by exon PCR (see Subheading 3.1.).
2. Ultrafree-MC centrifugal filter (Millipore, Tokyo, Japan).

Table 3
PCR Primer Sequences for Exon PCR^a

| Exon number | Primer sequences | Product length (bp) |
|-------------|---|---------------------|
| 1 | Forward: 5'-caggaacagctatgaccgcgcggtggcgccgctgcgcgca-3' Reverse: 5'-tgtaaacgacggccagtgcggcgagagaggctgtac-3' | 147 |
| 2 | Forward: 5'-caggaacagctatgaccatgttgctatcaccatttaaggg -3' Reverse: 5'-tgtaaacgacggccagtattggcagcgaggcgccatg-3' | 343 |
| 3 | Forward: 5'-caggaacagctatgaccacatgtcacttttgcttcct-3' Reverse: 5'-tgtaaacgacggccagttaggccatgtccatgcagactgc-3' | 462 |
| 4 | Forward: 5'-caggaacagctatgaccacaagcttttaagagtttctgt-3' Reverse: 5'-tgtaaacgacggccagttaggcaatgtgttagtacaca-3' | 296 |
| 5 | Forward: 5'-caggaacagctatgaccacatgtcttaaggagtacattt-3' Reverse: 5'-tgtaaacgacggccagttctctaattcctggcaaacagt-3' | 262 |
| 6 | Forward: 5'-caggaacagctatgaccagagattgttactgtggaaaca-3' Reverse: 5'-tgtaaacgacggccagtgaagtgtctatttttagatcct-3' | 303 |
| 7 | Forward: 5'-caggaacagctatgacctgcctttccacactgacaggctact-3' Reverse: 5'-tgtaaacgacggccagttctgttcttcattagcattagaga-3' | 274 |
| 8 | Forward: 5'-caggaacagctatgacctgatgtcataactgtgtgaag-3' Reverse: 5'-tgtaaacgacggccagtactgtctcattagcgtctatctt-3' | 241 |
| 9 | Forward: 5'-caggaacagctatgaccgggtgaaattgacagtcagt-3' Reverse: 5'-tgtaaacgacggccagtaataataatccagcccatgtgca-3 | 313 |
| 10 | Forward: 5'-caggaacagctatgaccattgccaaatgcaacctaatgtc-3' Reverse: 5'-tgtaaacgacggccagtttgaggaaatgagtagggcatt-3 | 200 |
| 11 | Forward: 5'-caggaacagctatgaccacagggaacataaaactctgatcc-3' Reverse: 5'-tgtaaacgacggccagtcaacacaccaggcacttcaga-3' | 338 |
| 12 | Forward: 5'-caggaacagctatgaccgtttgggaatgcgtgtttt-3' Reverse: 5'-tgtaaacgacggccagtgaattagaaaatgaaggtagaca-3' | 290 |

^aM13 universal and reverse sequences are underlined.

3. ABI Dye Terminator Cycle Sequencing Ready Reaction Kit (Perkin-Elmer).
4. M13 universal primer (5'-CAGGAAACAGCTATGACC-3' and M13 reverse primer (5'-TGTA AACGACGGCCAGT-3').
5. Loading buffer: deionized formamide and 25 mM EDTA, pH 8.0, in 50 mg/mL, 5/1 v/v.
6. Thermal cycler machine.
7. Sequence analyzer ABI 373.

2.3. Haplotype Analysis

1. Primers: These are the microsatellite markers covering the AR-JP locus and are listed in **Table 4**. Marker D6S437 is 3.0 cM apart from D6S305. Markers D6S305, D6S1579, D6S305, and D6S411 are located within 0 cM apart from each other. D6S253 is 5.0 cM apart from D6S305. These markers cover an 8.0 cM region

Table 4
Primers for Haplotype Analysis

| Marker | Primer sequences | Product length (bp) |
|---------|---|---------------------|
| D6S305 | Left: FITC-CACCAGCGTTAGAGACTGC Right: GCAAATGGAGCATGTCACT | 200–250 |
| D6S411 | Left: FITC-TGGTTGATTGACCCACTTAT Right: TCACAGTGCCTGGTCC | 150–200 |
| D6S1579 | Left: FITC-TACTCACACATGCACAGGC Right: CTCCTACCCACATGCAG | 100–200 |
| D6S437 | Left: FITC-TGTCCTGGTGGAGGCA Right: GGTACAGTGTGTTGACCCTAAGA | 100–200 |
| D6S253 | Left: FITC-GATCTGGGTTCACTTTGTC Right: GATACCAAGGGAAACTGG | 200–300 |

spanning the *parkin* gene. D6S305 is an intragenic marker, which is located in intron 7 of the *parkin* gene (5,6).

2. Electrophoresis buffer (10X TBE): 1 M Tris base, 0.83 M boric acid, 10 mM EDTA (filtered through a 0.45- μ m filter).
3. Polyacrylamide gel (0.5 mm thick) solution: 6% (w/v) acrylamine/bisacrylamide monomers (99:1), 100 mM Tris-borate (pH 8.3), 1 mM Na₂EDTA, and 7 M ALF grade urea filtered through a 0.22- μ m filter.
4. Ammonium persulfate: 10% (w/v) solution.
5. Tetramethyl ethylenediamine (TEMED).
6. Formamide loading dye: 100% deionized formamide and 5 mg/mL dextran blue 2000.
7. Sizer 50–500, 100, 200, 300 (Pharmacia): Fluorescein-labeled double-stranded DNA fragment (5 fmol/ μ L in TE buffer).
8. AmpliTaq DNA polymerase (Perkin-Elmer, Applied Biosystems Division).
9. 10 mM dNTPs.
10. 10X PCR buffer solution: 100 mM Tris-HCl at pH 8.3, 500 mM KCl, 15 mM MgCl₂, 0.01% gelatin.
11. Pharmacia ALF2 autosequencer.
12. Fragment manager software (Pharmacia)

3. Methods

3.1. Exon Deletions

1. Using primers shown in **Table 3**, prepare the following PCR mixture: 100–500 ng genomic DNA, 10 pmol each primer, 10 nmol dNTPs, 50 mM KCl, 10 mM MgCl₂, 0.01% gelatin, and 2.5 U Ampli Taq Gold DNA polymerase (Perkin-Elmer, Applied Biosystems Division) in 25 μ L.
2. Follow the PCR menus shown in **Table 5**. These should yield single PCR products (see **Note 5**).

Table 5
PCR Menus for Exon PCR

| |
|-------------------------|
| Exon 1 |
| Initial denaturation |
| 94°C for 10 min |
| 40 cycles of: |
| 96°C for 30 s |
| 60°C for 30 s |
| 72°C for 45 s |
| Final extension |
| 72°C for 10 min |
| Exons 2, 3, 6–9, and 10 |
| Initial denaturation |
| 94°C for 10 min |
| 40 cycles of: |
| 94°C for 30 s |
| 60°C for 30 s |
| 72°C for 45 s |
| Final extension |
| 72°C for 10 min |
| Exon 4 |
| Initial denaturation |
| 94°C for 10 min |
| 40 cycles of: |
| 94°C for 30 s |
| 53°C for 45 s |
| 72°C for 45 s |
| Final extension |
| 72°C for 10 min |
| Exons 5 and 12 |
| Initial denaturation |
| 94°C for 10 min |
| 40 cycles of: |
| 94°C for 30 s |
| 55°C for 30 s |
| 72°C for 45 s |
| Final extension |
| 72°C for 10 min |
| Exon 11 |
| Initial denaturation |
| 94°C for 10 min |
| 40 cycles of: |
| 94°C for 30 s |
| 62°C for 30 s |
| 72°C for 45 s |
| Final extension |
| 72°C for 10 min |

3. Electrophorese and visualize the PCR product on 2–3% agarose gel containing ethidium bromide (0.5 µg/mL).
4. Add a negative control sample with no DNA template in each experiment in order to exclude possible DNA contamination. Repeat the PCR studies at least twice to confirm the results.
5. When no exonic deletions are detected, proceed to exon sequencing.

3.2. Exon Sequencing

1. Following exon PCR (discussed above in **Subheading 3.1.**), use M13 universal and reverse primers for exon sequencing when no exonic deletions are detected.
2. Remove excess primers and dNTPs by using an Ultrafree-MC centrifugal filter (Millipore, Tokyo, Japan).
3. Perform the sequencing reaction according to the manufacturer's protocol for the ABI Dye Terminator Cycle Sequencing Ready Reaction Kit (Perkin-Elmer).

Sequencing Reaction mixture:

Terminator Ready Reaction Mix 8.0 µL
PCR template 100–200 ng
M13 universal or reverse primer 3.2 pmol
Add dH₂O to a final reaction volume of 20 µL

PCR conditions for the DNA Thermal Cycler are 25 cycles of:

96°C for 30 s
50°C for 15 s
60°C for 4 min

4. Purify the PCR products with Centri-Sep spin columns as described in the protocol supplied by the manufacturer.
5. Add the loading buffer, denature at 90°C for 2 min, chill on ice, electrophorese, and analyze the sequence with an ABI 373 Sequence Analyzer (*see Note 6*).

3.3. Haplotype Analysis

1. Label one of the primer pairs (sense or antisense primer) for microsatellite markers (**Table 4**) with fluorescein (FITC-labeled).
2. Prepare PCR mix: 10 µL reaction solution, 100 ng genomic DNA, 2.5 pmol of each primer, 2.0 nmol of dNTPs in 10 mM Tris-HCl, pH 8.3, 50 mM KCl, 1.5 mM MgCl₂, 0.001% gelatin, and 0.5 U AmpliTaq DNA polymerase.
3. Run the PCR menu as follows:
An initial denaturation for 5 min at 95°C, followed by 35 cycles of:
94°C for 0.5 min
50°C for 0.5 min
72°C for 0.5 min
A final extension at 72°C for 5 min.
4. Dilute the PCR product 10–20-fold with loading dye (*see Note 7*).
5. Add 5 fmol (1 µL) of 100-, 200-, and 300-bp fluorescein-labeled fragments (Sizer 100, 200, and 300 from Pharmacia), which encompass the size range of PCR

products to 3–4 μL of diluted samples (*see Note 8*). Sizer 50–500 (Pharmacia) is applied in one lane per 4–8 lanes and is used as an external standard (*see Note 8*).

6. Denature the samples at 94°C for 3 min.
7. Chill on ice.
8. Apply this mixture (5–6 μL) onto a 0.5-mm-thick 6% polyacrylamide gel with 0.6X TBE electrophoresis buffer.
9. Run the gel in 0.6X TBE electrophoresis buffer using a Pharmacia ALF2 fluorescence automated sequence analyzer.
10. Set the running condition at 1500 V, 38 mA, 34 W, and 45 Å. Set the Lazer power and interval at 3 mW power and 2 s, respectively.
11. After running the gel, analyze the PCR products by Fragment manager software (Pharmacia) (*see Figs. 3 and 4*).

4. Notes

1. A recessive disease is caused by the presence of two mutations, each of which has occurred in the same gene residing on homologous chromosomes. A compound heterozygote is a patient who has two different mutations on each of homologous chromosomes. As each of the mutations is derived from a different ancestor of the disease mutation, a compound heterozygote has two different haplotypes (*see Note 8*), which originate from different ancestors of the mutation.
2. A haplotype is a set of alleles on one chromosome. Alleles are alternative forms of a gene or marker occupying the same locus on homologous chromosomes. As human cells have two copies of each chromosome (diploid cells), an individual always has a pair of alleles, one from each parent. Accordingly, an individual has two haplotypes. If alleles are very closely linked, haplotypes within a kindred are transmitted as units. However, when alleles are not closely located, recombination by crossing over occurs and haplotypes are changed. Homozygotes have the same alleles or haplotypes on both homologous chromosomes, whereas heterozygotes have different alleles or haplotypes.
3. In a consanguineous pedigree, each parent is usually a carrier of the same mutation, i.e., has a single identical mutation derived from a single person who first acquired the mutation in an earlier generation, i.e., the ancestor of the mutation. Accordingly, if a patient born from a consanguineous marriage has homozygous haplotypes for the markers that flank or reside in a certain gene, this is a strong indication that the patient has two identical mutations in the same gene (theory of homozygosity mapping) (*18*). The probability for homozygosity to show true linkage is heavily dependent on the rarity of the alleles or haplotypes showing homozygosity. This is based on the fact that if the frequency of the marker in the control population is rare, the chance for its heterozygosity in the general population as well as in the parents increases. The latter, in turn, increases the power of detection for the single identical allele to be transmitted from each parent to the affected person (homozygosity by descent). On the other hand, if the marker frequency is high in the general population, homozygosity by chance increases and, therefore, homozygosity in the patient by itself is not informative (homozygos-

ity by state). Thus, to substantiate segregation of the haplotype with the disease, especially when only information about the patients' haplotypes is available, knowledge of the allele frequencies of the markers that constitute homozygous haplotypes are important. Analysis of 30–50 DNA samples obtained from normal persons is sufficient to determine allele frequencies of the markers.

4. PCR with primers without M13 universal sequences are also possible. In this case, the extracted DNA can be directly sequenced using internal primer sequences or can be subcloned into the TA-vector plasmid (TA-vector cloning kit, Invitrogen) without filling in the ends of the DNA fragment. The insert in the TA-vector can be sequenced with universal primers (M13 and M13 reverse). Several clones should be assessed to exclude possible PCR- and cloning-based mutations.
5. If extra bands in PCR products are observed in the gel, cutting the band corresponding to the expected size, extraction, and purification of DNA with the Qiaquick Gel extraction kit (Qiagen) is recommended for further sequencing.
6. Single-strand sequencing using T7 polymerase is an alternative method for the sequencing. The major merit of single-strand sequencing with T7 polymerase is uniformity of signal intensity, allowing easy detection of heterozygous mutations. The sequencing kit (Autoread sequencing kit) can be purchased from Pharmacia (Uppsala, Sweden). The single-strand template is recovered from the PCR product by magnetic force. As one of the PCR primers is biotin-labeled, the addition of streptavidin-coated magnetic beads (Dyna) to the PCR product results in their binding to the biotin-labeled DNA strand. Accordingly, the biotin labeled strand is isolated by magnetic force. A sequencing sample is applied in four lanes (A, C, G, and T) of the sequencing gel and analyzed with a Pharmacia ALF2 fluorescence autosequence analyzer. Universal sequences are added to the 5' end of PCR primers and fluorescein isothiocyanate (FITC)-labeled universal primers are used for sequencing. FITC-labeled universal primers are included in the Autoread sequencing kit (5'-CGACGTTTAAAACGACGGCCAGT-3' for M13 primer and 5'-CAGGAGGCAGCTATGAC-3' for M13 reverse primer). Sequencing primers must be derived from the region located at least one nucleotide internal to the site of PCR primers.
7. Scale-out of the peak of the signal occurs when dilution of the sample is insufficient.
8. Two different types of size standards—internal and external—are used. As internal standards, two size markers encompassing the size of the PCR product are loaded in the same lane with the PCR product. For example, Sizers 200 and 300 are loaded with the product whose expected size is between 200 and 300 bp. The molecular size of the peak of the PCR product is determined by reading the retention times of respective peaks of internal standards flanking the PCR product. As external standards, only the sizer markers are loaded in the lane that is called as the reference lane. For each group of sample lanes (usually four to five lanes) with reference lanes on both sides, the standard curves of the reference lanes are calculated. The molecular size of the PCR sample is calculated by first using the external standard and then adjusting the resulting standard curves to the internal reference points.

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