Molecular Findings in Familial Parkinson Disease in Spain

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**Background:** Several genetic errors in α-synuclein (Park1) and ubiquitin carboxyl-terminal–hydrolase L1 (Park5) genes cause autosomal dominant familial Parkinson disease. Mutations in the parkin gene (Park2) are the major cause of autosomal recessive Parkinson disease.

**Objective:** To analyze the clinical and molecular data of 19 Spanish kindreds (13 with recessive, 4 with dominant, and 2 with uncertain inheritance) who have familial Parkinson disease.

**Methods:** We searched for the previously described mutations in Park1 and Park5 genes and for new or described mutations in Park2. We used single-strand conformation polymorphism, direct sequencing, and restriction digestion of polymerase chain reaction (PCR)–amplified genomic DNA for this study.

**Results:** None of these families have either Park1 or Park5 mutations. We found 5 different mutations in Park2 gene in 5 of the families with recessive inheritance. To our knowledge, 2 of these mutations, V56E and C212Y, have not been previously reported. The other mutations found (deletion of exons 3 and 5 and 225delA) have been described in other ethnic groups. Heterozygous carriers of a single Park2 mutation either were asymptomatic or developed clinical symptoms in late adulthood or after brief exposure to haloperidol therapy.

**Conclusions:** Mutations in Park2 gene account for 38% of the families with recessive parkinsonism in Spain. We found 2 cases of simple heterozygous Park2 mutation carriers that developed clinical symptoms, either in late adulthood or after brief exposure to parkinsonizing agents. Thus, hereditary Parkinson disease has more variable clinical phenotype and molecular defects than previously thought since heterozygous mutations could be a risk factor for parkinsonism.

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**Parkinson Disease (PD)** (OMIM 168600) is a common neurologic disorder of unknown origin in most cases, although several families with monogenic inheritance have been reported. Two mutations in the α-synuclein gene (Park1), Ala53Thr and Ala30Pro, and 1 mutation of the ubiquitin carboxyl-terminal–hydrolase-L1 gene, Ile93Met, cause autosomal dominant PD in a few families.

Autosomal recessive juvenile parkinsonism (ARJP) (OMIM 600116) is a distinct clinical and genetic entity within familial PD. Autosomal recessive juvenile parkinsonism is characterized by onset before age 40 years, good response to levodopa therapy, prominent fluctuations, absence of Lewy bodies, and presence of neurofibrillary degeneration in the substantia nigra. The gene for this disorder, mapped to chromosome 6q26-q27 and named parkin, was identified in Japanese families with ARJP. Around 50% of familial ARJP and 18% of sporadic PD in Europe are caused by mutations in the parkin gene (Park2) (OMIM 602544): exon deletions, exon duplications, and point mutations.

Parkin protein is an E3 ubiquitin-protein ligase with an ubiquitinlike (Ubl) domain at its NH2-terminus. In its COOH-terminus there are 2 RING-finger motifs and an IBR (in between RING fingers), organized as a RING-IBR-RING domain. Thus, patients with parkin mutations could have an abnormal ubiquitination pathway.

In this study, we performed the molecular analysis of 19 Spanish families with PD. Five families with recessive inheritance have different mutations of Park2 gene, among them, V56E and C212Y. To our knowledge, V56E and C212Y are reported for the first time. In addition, we found 2 cases of simple heterozygous mutation in individuals that developed clini-
PATIENTS AND METHODS

CLINICAL ANALYSIS

In this study we included 19 unrelated families with PD from different regions of Spain. 18 families of Spanish origin and 1 of Lebanese origin. Thirteen of these families have recessive inheritance (families 1-5, 8, 9, 11-19, and 18), 4 have dominant (families 6, 7, 10, and 16), and 2 have uncertain inheritance (families 17 and 19). All patients were personally examined by one of us (J.G.Y.) and most of them by other members of the research team. The assignment of the clinical phenotype was performed in the Movement Disorders Clinics of their referring hospitals or in their residences. The clinical investigation included personal and familial clinical history, clinical examination findings, quantitative motor examination results, and a videotaped examination. The individuals were considered affected when they fulfilled the clinical criteria of the slightly modified London Brain Bank. We required that all patients should have aminexia. Asymmetry of the initial symptoms and good response to levodopa therapy were considered positive for the diagnosis of PD. Informed consent was obtained from patients, members of their families, and control subjects.

MOLECULAR ANALYSIS

High molecular weight genomic DNA, purified from peripheral blood leukocytes according to standard methods, was used as a template for polymerase chain reaction (PCR)–based methods of molecular analysis. We looked for the previously reported missense mutations in Park1 and Park2 genes. The screening of these mutations was performed as previously described. Restriction digestions of PCR products were done according to the manufacturer’s protocol (New England Biolabs Inc, Beverly, Mass). Plasmid DNA was used as a positive control in α-synuclein gene analysis and the restriction maps obtained were compatible with the known sequence.

The study of Park2 gene was performed by linkage analysis to chromosome 6q26-q27, single-strand conformation polymorphism (SSCP) screening, and direct sequencing. Genotyping was carried out according to standard protocols (ResGen, an Invitrogen Corp, Huntsville, Ala; also available at: http://www.resgen.com) with the following short tandem repeat polymorphisms: D6S437, D6S1035, D6S1579, D6S1550, D6S305, D6S411, and D6S955. The results were analyzed with the MLINK program of the LINKAGE package, Version 5.2 for the linkage analysis. The penetrance was assumed to be 100% for a recessive model of inheritance. The coding region of Park2 gene was amplified using the same oligonucleotides primer pairs as previously described. The reactions were performed in a 25-µL reaction mixture containing 50M potassium chloride, 10M Tris buffer, pH, 8.3; 1.75M magnesium chloride; 25 pmol of paired primers; 10 nmol of each deoxynucleoside triphosphate; and 1.25 U of AmpliTaq Gold DNA polymerase (Perkin-Elmer, Applied Biosystems Division, Foster City, Calif.). Following initial denaturation at 94°C for 10 minutes, amplification was performed through 35 cycles of 94°C for 30 seconds, 51°C for 30 seconds, and 72°C for 45 seconds, and then a final extension at 72°C for 10 minutes.

All amplified exons were screened by SSCP analysis using the Genephor System (Amersham Biosciences Europe GmbH, Barcelona, Spain) at 15°C according to the manufacturer’s recommendations. Patients’ samples exhibiting band shifts on SSCP gels were subjected to direct sequence.

Families 11 and 18 were excluded from the SSCP screening because they carried homozygous deletion of exon 3 and 5, respectively. To confirm both deletions we designed the following inner primers: 3 inner forward 5’-AGCAG AGCATGTTCCATGG-3’ (108 downstream from the 3 forward primer), 3 inner reverse 5’-ACTCCAGCTGTT GGTGAG-3’ (54 nucleotides upstream of the reverse primer), 5 inner forward 5’-TCCATCTTG CTGGGATGATG-3’ (46 nucleotides downstream from the 5 forward primer), and 5 inner reverse 5’-TCCAATAAGAG GAATGAATGG-3’ (18 nucleotides upstream of the 5 reverse primer). The same primers were used for amplification and sequencing the PCR-amplified fragments using fluorescence-based automated sequencing technique (Applied Biosystem 373a DNA sequencer; Perkin-Elmer)

MUTATION SCREENING IN THE SPANISH POPULATION

When it was possible, the co-segregation of certain mutations with the disease was established by restriction endonuclease digestion of PCR products in the patient’s family. The frequency of these mutations in the Spanish population was determined in 48 individuals without neurologic diseases. Digestion conditions were in accord with the manufacturer protocols. After digestion, fragments were electrophoresed in 3% low–melting point agarose, stained with ethidium bromide, and visualized under UV light.

SOUTHERN BLOT ANALYSIS

Ten micrograms of genomic DNA from patients and controls were digested with EcoRI and PstI restriction enzymes subjected to electrophoresis, and blotted onto a nylon membrane (Hybond N+; Amersham Biosciences Europe GmbH) by standard procedures. The membrane was hybridized with parkin complementary DNA (nucleotides 290-1291) labeled with [32P]deoxycytosine triphosphate, by use of a Prime-It RmT Random Primer labeling kit (Strategene, Amsterdam, the Netherlands).
family 18 (Figure 1B). We confirmed this finding through a second PCR with inner set of primers, co-amplification with a control target and Southern blot analysis (Figure 1C and Figure 2). The other deletion, 255delA, present in families 3 and 13 (Figure 3), has already been described in other European patients. Patients from family 3 were homozygous for 255delA while, in family 13, the mutation appears in only 1 allele. The other mutant allele in family 13 consists of a heterozygous exons 8 and 9 deletion. The lesion was inferred by the alleles pattern obtained for marker D6S411 in the individual EP13.3 and confirmed by Southern blot (Figure 3B and Figure 2).

Two novel disease-causing mutations were found in 3 affected individuals of family 4 (Figure 4A). V56E consists in a T → A transversion at position 268 in the complementary DNA that creates a new Bst4CI restriction site. This mutation changes the nonpolar valine residue in position 56 for the acidic polar glutamic residue (Figure 4B). C212Y is a 736 G → A transition located in exon 6 (Figure 4C) that replaces a highly conserved cysteine for the large aromatic amino acid tyrosine. The 3 patients were compound heterozygotes for these 2 mutations. The mendelian inheritance was confirmed for C212Y, which is carried by the father. Both mutations were screened in 96 control chromosomes. Neither the abnormal SSCP pattern corresponding to C212Y nor the new Bst4CI restriction site, created for V56E, were found in any control. Segregation analyses showed that the 2 mutations are located in different alleles (Figure 4A).

The clinical phenotype in the affected individuals from families with known Park2 gene defects was typical of ARJP with some exceptions. One member of family 3 (Figure 3A), a single heterozygous carrier of 255delA, developed transient drug-induced parkinsonism at 45 years of age while being treated with haloperidol, 2 mg/d. Another individual, a member of family 4 (Figure 4A), a single heterozygous C212Y carrier, developed clinical symptoms at 78 years of age, while 3 of his sons with this mutation plus V56E noted that the disease started at ages 33, 33, and 27 years.

**COMMENT**

We studied the 3 genes involved in familial PD. None of the studied families have Park1 gene mutations suggesting that, even in Mediterranean countries, mutations in the /H9251-synuclein gene are a rare cause of PD. We also failed to find Ile93Met mutation in ubiquitin carboxy-terminal–hydrolase L1 gene in our families.

The molecular analysis of Park2 gene allowed the molecular characterization of 5 families, 2 with homozygous deletions of 1 exon, 2 with a truncating mutation, and 1 with 2 combined heterozygous novel missense mutations. The new point mutations, V56E in exon 2 and C212Y in exon 6, lie in different parkin functional domains. The nonconservative V56E amino acid change, in the Ubl domain of parkin, is located in a conserved
position among ubiquitins from phylogenetically distanced organisms Rattus norvegicus (swall: Q9JM64), Mus musculus (swall: AA613892), Drosophila melanogaster (swall: Q9VP72), and Tetrahymena pyriformis (swall: Q27194). Since the Ub domain is required for the recognition of the ubiquitinated proteins, ParkinV56E could be unable to bind with its target protein, which might result in pathological accumulation of the target and negative effects on the central nervous system.

C212Y is located in a highly conserved residue and very close to the first RING-finger motif that is required for E3 ubiquitin-protein ligase activity of parkin. This region contains up to 8 conserved cysteine residues that may be involved in disulphide bridges and, therefore, a cysteine substitution could change the secondary structure of the protein besides the first RING finger.

Regarding the 255delA mutation, present in families 3 and 13, we found by the analysis of microsatellites that the deletion lies in 3 different haplotypes (Figure 3). The sequence analysis of the region around 255delA showed the presence of short direct repeats at the 3' region, suggesting that a recurrence mechanism is involved.

Three patients with early-onset PD from family 4 are combined heterozygous for V56E and C212Y. One brother, 45 years old, a heterozygous carrier of the mutation V56E, is asymptomatic. The father, however, a single heterozygous carrier for C212Y mutation, developed parkinsonism at the age of 78 years. Recently, Klein

Figure 3. Pedigrees of families with 255delA mutation. A, Family 3. B, Family 13 with genotypes for some markers. Squares indicate men; circles, women; solid symbols, affected individuals; open symbols, unaffected individuals; and slashed symbols, deceased individuals. bp indicates base pair; N, normal allele; and question mark, unknown allele. Gray filled symbol indicates drug-induced parkinsonian individual. Bars represent the haplotypes.

Figure 4. A, Pedigree of family 4 showing the genotype for different single-nucleotide polymorphisms. Markers in parentheses were inferred from other family data. Haplotypes are shared by the members of each family are represented by the bars. B and C, Tracings for exons 255delA/?
et al. identified, in a large family, heterozygous patients with Park2 mutations in 1 allele clinically indistinguishable from most patients with idiopathic PD. Moreover, positron emission tomographic analysis in this family indicates a preclinical disease process in the asymptomatic heterozygous carrier subjects. Our data agree with those findings and expand our knowledge about the influence of Parkin abnormalities in PD.

In addition, we have evidence that heterozygous mutations in Park2 could be a risk factor for drug-induced parkinsonism. Eight members of family 3, heterozygous carriers of the 255delA mutation, are asymptomatic, but 1 of the 8 developed parkinsonism when treated with haloperidol, 2 mg/d. This patient fully recovered after discontinuation of the drug. Neuroleptic-induced parkinsonism is more frequent in relatives of patients with PD and that increased risk has never been explained. Thus, it is conceivable that individuals with half activity of E3 ubiquitin-ligase parkin as heterozygous carriers could be more susceptible to drug-induced parkinsonism, to the effects of aging, or to environmental agents. These findings, which could be overlooked as merely anecdotal, need to be investigated in a larger and specifically designed study. The role of parkin in sporadic PD remains to be discovered, but our observations suggest that Park2 might be implied in the origin of the more frequent late-onset PD and drug-induced parkinsonism.

The study of whether the heterozygous state predisposes to disease will help explain parkin implications, if any, in common sporadic PD and drug-induced parkinsonism either in late adulthood or after a brief exposure to parkinsonizing agents.

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