

α -Synuclein Gene Rearrangements in Dominantly Inherited Parkinsonism

Frequency, Phenotype, and Mechanisms

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Objective: Genomic multiplications of the α -synuclein gene (*SNCA*) cause autosomal dominant Parkinson disease (ADPD). The aim of this study was to assess the frequency and phenotype of *SNCA* rearrangements in a large series of families with typical or atypical AD parkinsonism.

Design: Patients were screened by the exon dosage of the *SNCA* gene. The genotype of patients and relatives carrying *SNCA* rearrangements, the size of the multiplied regions, and the centromeric and telomeric break-points were determined by microsatellite dosage and 250K Affymetrix Single Polymorphism Nucleotide microarrays (Affymetrix, Santa Clara, California).

Subjects: Index cases and, whenever appropriate, relatives of 286 mainly European families with ADPD were screened.

Results: Four of 264 families (1.5%) with typical ADPD carried duplications and 1 of 22 families (4.5%) with atypi-

cal AD parkinsonism carried a triplication of *SNCA*. Genotyping and dosage analyses showed that the multiplied regions were variable in size (0.42-5.29 megabase pairs), suggesting that *SNCA* multiplications occurred independently. Phenotype analyses showed that the severity of the disease correlated with the *SNCA* copy number, but not with the minimal number of multiplied genes (1 to 33). Haplotype analysis of polymorphic markers suggested that multiplication of the *SNCA* gene occurred by both interchromosomal and intrachromosomal rearrangement.

Conclusions: Our results suggest that *SNCA* rearrangements may be more frequent than point mutations in ADPD. Furthermore, our results indicate that the phenotype associated with *SNCA* multiplications correlates with the number of copies of the gene and provides the first insight into the mechanisms underlying *SNCA* multiplication.

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PARKINSON DISEASE (PD) (OMIM 168600) is a frequent progressive neurodegenerative disorder. Symptoms are caused mainly by loss of dopaminergic neurons in the substantia nigra. However, the mechanisms by which these neurons degenerate remain unknown. The study of familial forms of the disease compatible with mendelian inheritance and accounting for less than 10% of PD cases¹ has led to the identification of 11 genes and/or loci implicated in the pathology.² Polymeropoulos et al³ identified the first mutation causing PD (p.A53T) in the α -synuclein gene (*SNCA*; OMIM 163890) in a large Italian American family with autosomal dominant parkinsonism. After intensive screening of the *SNCA* gene in patients with PD of different origins, only 2 additional point mutations have been identified (p.A30P⁴ and p.E46K⁵), suggesting that point mutations in *SNCA* are a rare

cause of PD. In contrast, different duplications and triplications of the whole *SNCA* gene are found in families with autosomal dominant forms of the disease.⁶⁻¹¹ More recently, *SNCA* duplications were also identified in rare, apparently sporadic cases.^{12,13} The severity of the phenotype, ranging from typical PD to dementia with Lewy bodies (DLB); (OMIM 127750) and PD dementia, and the brain structures affected have been shown to increase with the number of copies of the gene. Most *SNCA* duplication carriers (3 copies of the gene) have typical late-onset parkinsonism,⁶⁻⁹ whereas those with triplications (4 copies of the gene) have earlier-onset and atypical parkinsonism.^{10,11} However, owing to the limited number of studies, the prevalence and mechanisms leading to these rearrangements have not been precisely determined.

The aim of this study was to assess the frequency of *SNCA* rearrangements in a large series of families with typical or atypi-

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cal parkinsonism compatible with autosomal dominant inheritance and to determine the mechanism by which they occur.

METHODS

PATIENTS

In this study, 345 patients with parkinsonism from 286 families (including 1⁹ and 119⁸ already published) compatible with autosomal inheritance were examined by a member of the French Parkinson's Disease Genetics Study Group. A total of 210 families had at least 1 affected parent-child pair, and 76 families had at least 2 affected relatives in 2 different generations (uncle-aunt and/or nephew-niece pairs). In addition, 37 of the 76 index cases had an affected sibling. Diagnostic criteria for definite PD were the presence of at least 3 of the following cardinal signs: resting tremor, bradykinesia, rigidity, and good (>30%) response to levodopa therapy. In 264 of the families, exclusion criteria such as ophthalmoplegia, dementia, and dysautonomia early in the course of the disease, as well as apraxia, cerebellar, and pyramidal syndromes were absent. The clinical features of the 3 families carrying duplications (FPD-131,⁹ FPD-321, and FPD-410⁸) have already been published. In addition, we screened 22 index cases with atypical parkinsonism. These patients had 1 or more atypical symptoms including pyramidal signs (n=8), early dementia (n=7), myoclonus (n=2), apraxia (n=2), supranuclear ophthalmoplegia (n=2), hypoventilation (n=1), axonal neuropathy (n=1), dysautonomia (n=1), no response to levodopa treatment (n=2), cerebellar ataxia (n=1), and subacute onset (n=1).

The mean (SD) age at onset of disease in index patients (140 women; 146 men) was 48.6 (13.2) years (range, 8-86 years) and at examination was 57.3 (13.0) years (range, 25-88 years). The 286 families were mostly of European descent (n=259), including families from France (n=230), Italy (n=11), Spain (n=4), Germany (n=3), the United Kingdom (n=3), Portugal (n=3), the Netherlands (n=1), Ireland (n=1), Greece (n=1), and Eastern Europe (n=2). There were also patients from North Africa (n=15), South (n=2) and North America (n=1), the West Indies (n=5), Turkey (n=1), Asia (n=1), and the Middle East (n=2).

Local ethics committees approved the study and written informed consent was obtained from all participants. Peripheral blood was collected from each patient; genomic DNA was extracted from leukocytes according to standard procedures and was used for subsequent molecular analyses.

MOLECULAR ANALYSES

Detection of SNCA Rearrangements

Dosage of the SNCA gene was performed by semiquantitative multiplex polymerase chain reaction (PCR), as previously described.⁸ Briefly, exons 3 and 4 of this gene were coamplified with 2 internal controls: a 236-base pair sequence of a reference gene, transthyretin (OMIM 176300), and the exon 4 of the parkin gene (OMIM 602544). Two diploid subjects were used as negative controls and a patient with a known heterozygous deletion of parkin exon 4 as positive control. The PCR products were quantified using GeneMapper 3.5 software (Applied Biosystems, Foster City, California) on an ABI 3730 genetic analyzer (Applied Biosystems). The number of copies of the gene was determined from the ratios of mutant to control alleles compared with ratios obtained from DNA of the control subjects. Ratios between 0.8 and 1.2 indicated a normal

individual; between 1.3 and 1.7, a heterozygous duplication; and between 1.8 and 2.2, a triplication or homozygous duplication. Multiplied regions were further validated with intronic and flanking microsatellite markers in available members of positive families as previously reported⁸; probands were tested for the LRRK2 G2019S mutation. All tests were performed at least twice.

Refinement of the Multiplied Regions

To determine the extent of the multiplied region in each positive family, we analyzed 23 existing and 13 newly developed quantitative microsatellite markers spanning approximately 8 megabase pairs (Mb) within the 4q21.3 to 4q22.3 genomic region (primer sequences available on the Genome Database human genome database Web site; <http://www.gdb.org>).

To pinpoint the breakpoint ends of the multiplied regions spanning SNCA, Affymetrix GeneChip Human Mapping 250K Nsp arrays genotyping and single-nucleotide polymorphism (SNP) dosage analysis were performed according to the manufacturer's protocol (Affymetrix, Santa Clara, California). Affymetrix GCOS v1.4 software was used to obtain raw microarray feature intensities (Raw microarray feature intensities [RAS] scores; raw data available on request). The RAS scores were processed using Affymetrix GTYPE v4.0 software to derive SNP genotypes. Copy number estimates were obtained using the program dChip.¹⁴⁻¹⁶ Raw copy number estimates were then smoothed using a Hidden Markov Model approach that treats copy number along the chromosome as a Markov process with a very low probability of switching between different copy number states.

The minimal size of the multiplied regions was defined by the last informative multiplied microsatellite or SNP marker and the maximal size extended up to the first informative but not multiplied marker on each boundary of the SNCA gene.

Bioinformatics Analyses

Information available from the Ensembl Genome Browser (<http://www.ensembl.org>), the University of California Santa Cruz Genome Browser (<http://genome.ucsc.edu/>), the National Center for Biotechnology Information Web site (<http://www.ncbi.nlm.nih.gov/>), and the Genome Database Web site (<http://www.gdb.org>) was used to design PCR primers and to identify known and predicted genes in the multiplied regions. Repeat elements and low-copy repeats at the centromeric and telomeric boundaries of each multiplied region were searched for using the RepeatMasker (<http://www.repeatmasker.org/>) and BLASTN (<http://www.ncbi.nlm.nih.gov/blast/>) programs. For this purpose, genomic sequences of interspersed sequence repeats that had been filtered out were compared with the complete human sequence genome draft.

Haplotype Analysis

To identify alleles shared among and within families and to further analyze the mechanism of the rearrangement in each family, haplotypes for all available members of families with SNCA gene multiplications were constructed manually, allowing a minimum number of recombinations. We used 7 microsatellite markers (NACP-REP1 and 6 newly developed markers) located within the shared SNCA-containing region. Flanking markers NACP-REP1 and D4S3460 are located approximately 9 and 10 kilobase pairs (Kb) from the 5' and 3' ends of the SNCA gene, respectively. The D4S3455 and D4S3456 markers are located in introns 1 and 2, respectively, with D4S3457, D4S3458, and D4S3459 in intron 3 of the SNCA gene.

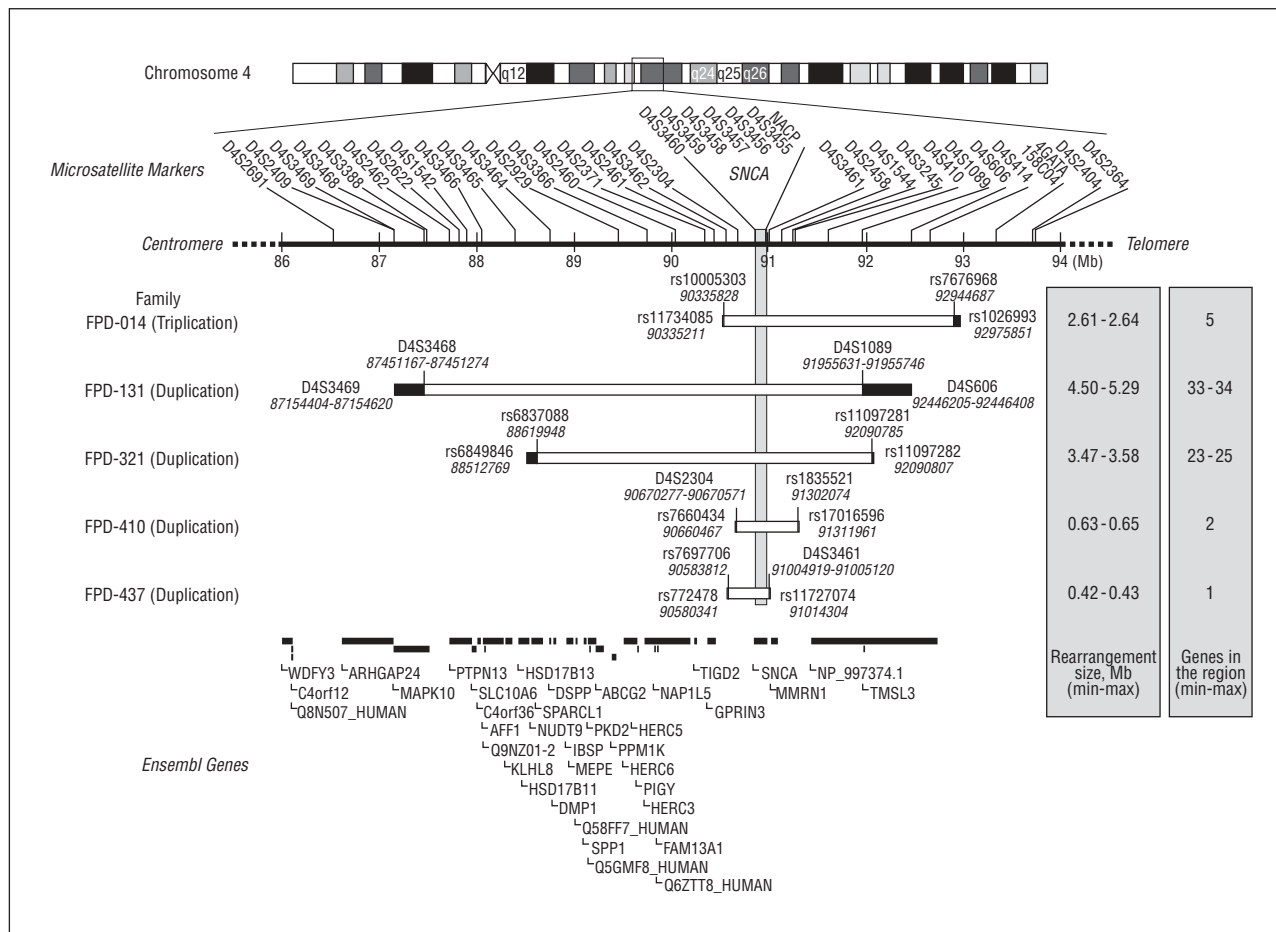


Figure 1. Breakpoint analysis of 5 families with *SNCA* multiplications. Mb indicates megabase pairs; min, minimum; and max, maximum.

Statistical Analysis

Comparisons between patient groups were made with the χ^2 test or the Fisher exact test when appropriate for qualitative variables and analyses of variance for quantitative variables using SPSS Software (SPSS Inc, Chicago, Illinois).

RESULTS

DETECTION AND REFINEMENT OF THE MULTIPLIED REGION CONTAINING *SNCA*

Using semiquantitative multiplex PCR to detect exon rearrangements, we found *SNCA* duplications in 4 families and a triplication in 1, but no deletions. The presence of extra copies of the whole *SNCA* gene in these families was confirmed by analyzing 2 flanking and 7 intragenic *SNCA* microsatellite markers. The families were from France (FPD-014, FPD-131, FPD-410, FPD-437) and Italy (FPD-321). All affected members of these families whose DNA was analyzed were heterozygous carriers of an *SNCA* multiplication. Analyses of 36 polymorphic microsatellite markers confirmed these results and allowed estimation of the size of the rearrangements. Refinement of the multiplied regions sizes in 4 of the 5 families was performed by dosage analysis using Affymetrix 250K SNP microarrays. In the remain-

ing family (FPD-131), the duplication was confirmed, but the boundaries could not be refined because no more genomic DNA was available for analysis. The minimal size of the multiplied regions differed among the families and ranged from 0.42 to 4.50 Mb, suggesting that the events occurred independently (**Figure 1**). Family FPD-014 had a triplicated region of 2.61 to 2.64 Mb that contained 4 known genes (*SNCA*, multimerin 1 [*MMRN1*] [OMIM 601456], thymosin-like 3 [*TMSL3*; NM_183049], G protein-regulated inducer of neurite outgrowth 3 [*GPRIN3*] [OMIM 611241]), and a gene encoding the hypothetical protein LOC401145 (NP_997374.1; NM_207491). Members of family FPD-131 had the largest duplication (4.50-5.29 Mb), according to microsatellite dosage, which contained 33 to 34 genes (Figure 1). Several of these genes encode mitogen-activated protein (MAP) kinases or phosphatases; 3 others encode members of the HECT domain and RCC1-like domain family (*HERC1*) of ubiquitin ligases. Five of these genes, the MAP kinase 10 (*MAPK10*) (OMIM 602897), *SNCA*, *MMRN1*, polycystic kidney disease 2 (*PKD2*) (OMIM 173910), and dentin matrix acidic phosphoprotein (*DMP1*; OMIM 600980) genes have been associated with human diseases. The duplicated region in family FPD-321 was the second largest (3.47-3.58 Mb) and contained at least 23 genes. The duplicated region in family FPD-410 (approximately

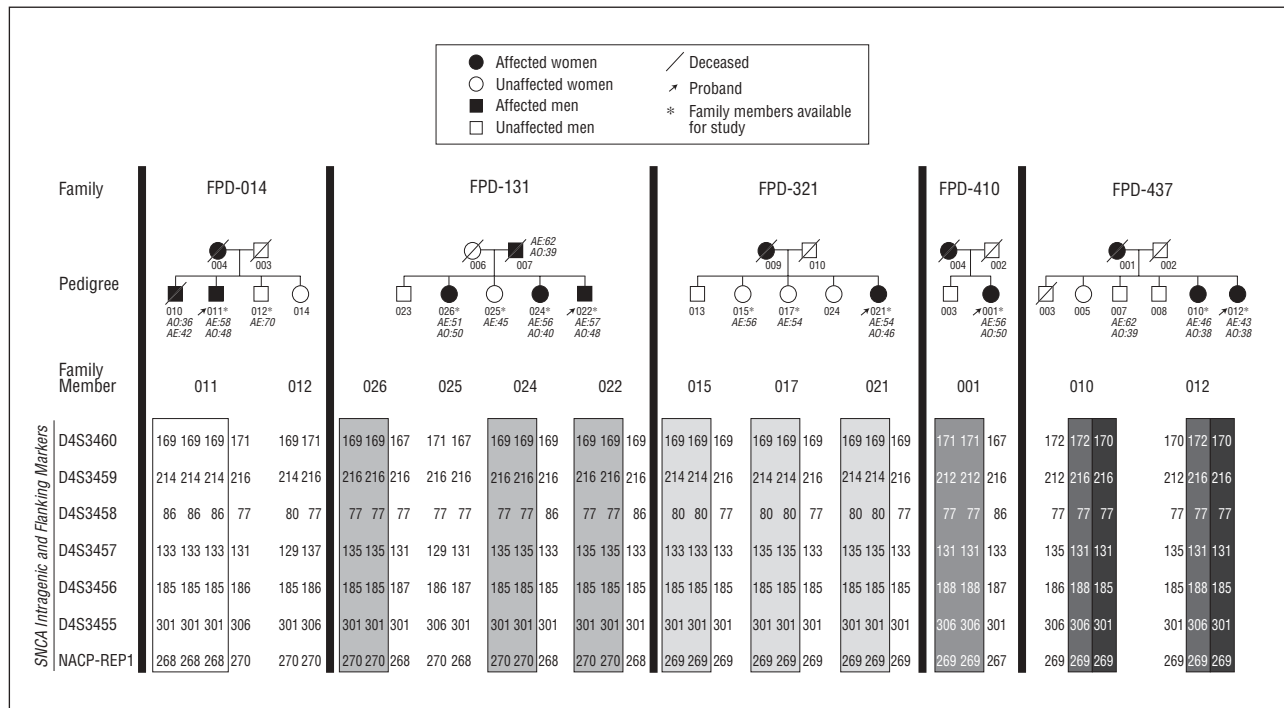


Figure 2. Duplications and triplications of the *SNCA* gene caused by intrachromosomal or interchromosomal rearrangements.

0.63-0.65 Mb) contained *SNCA* and *MMRN1*. The duplication in family FPD-437 (approximately 0.42-0.43 Mb) contained only *SNCA*.

RepeatMasker software revealed a variable number of interspersed repetitive elements, including Alu elements, long interspersed nuclear elements, and long terminal repeats in both the centromeric and telomeric boundaries of the multiplied regions containing *SNCA* in all families except FPD-321 and FPD-014. No low-copy repeats, 10- to 400-Kb DNA blocks with more than 95% identity between copies, were found in the intervals containing the breakpoints.

HAPLOTYPE ANALYSIS

Analysis of 7 microsatellite markers within the *SNCA* multicopy region showed that each family had a different set of alleles, confirming that the mutations were not inherited from a common ancestor (**Figure 2**). In families FPD-131, FPD-321, and FPD-410, all *SNCA* duplication carriers had 2 identical copies of the region (**Figure 2**). Similarly, the *SNCA* triplication carrier FPD-014-011 had 3 identical copies of the region plus a haplotype of the region that he shared with his unaffected brother. In contrast, both duplication carriers from family FPD-437 shared a duplicated region containing 2 haplotypes, but the haplotype of the third copy of the region was not shared (**Figure 2**).

CLINICAL CHARACTERISTICS

All families with duplications had typical PD, whereas the family with a triplication had atypical features. Patients with duplications and triplications of the *SNCA* gene had similar ages at examination, ages at onset, and dis-

ease durations (**Table**). In the 4 families with *SNCA* duplications, all patients had typical PD that was similar to the phenotype of patients with typical autosomal dominant PD (ADPD) without *SNCA* rearrangements. Two *SNCA* duplication carriers were still asymptomatic at ages 56 and 54 years (FPD-321-015 and FPD-321-017; **Figure 2**). By 8 and 10 years of age, respectively, both were older than the age at onset of their affected sister (FPD-321-21) whose disease began at 46 years but had not reached the latest age at onset in patients with duplications in this series, which was 65 years. These observations were consistent with incomplete age-dependent penetrance.

The patients carrying *SNCA* triplications had atypical parkinsonism with rapidly evolving symptoms and severe cognitive impairment. Their response to levodopa treatment was limited at onset or decreased very rapidly after onset. Triplication carriers had shorter disease durations until death than duplication carriers (mean [SD] disease duration, 7.0 [2.6] years; range, 5-10 years; n=3 vs 10.5 [7.2] years; range, 1-23 years; n=3), assuming that patients who could not be analyzed for gene dosage carried the multiplication. Owing to the small number of cases, however, the difference was not statistically significant. Despite shorter disease durations, patients with triplications had more severe dementia (mean [SD] Mini-Mental State Examination score, 15.0 [12.8]; range, 6-24; n=2 vs 27.8 [2.0]; range, 26-30; n=5; $P=.05$), were more severely affected (mean [SD] Unified Parkinson Disease Rating Scale motor score during treatment, 44.5 [2.1]; range, 43-46; n=2 vs 11.7 [6.1]; range, 5-17; n=3; $P=.006$), and had urinary incontinence more frequently (3/3 vs 0/6; $P=.01$) than those with duplications.

Table. Clinical Comparison of Patients With PD With Typical or Atypical Presentations and With SNCA Duplications or Triplications^a

Characteristics	SNCA Triplication (n=3, 1 Genotyped)		SNCA Duplication (n=9, 7 Genotyped)		P Value	Atypical Parkinsonism (n=24)		Typical Parkinsonism (n=313)	
	Mean (SD)	Range (Available Data ^b)	Mean (SD)	Range (Available Data ^b)		Mean (SD)	Range (Available Data ^b)	Mean (SD)	Range (Available Data ^b)
Sex, women:men		1:2		7:2	.24		16:18		155:158
Age, y	55.3 (12.2)	42-66	56.6 (11.8)	43-84	.88	56.8 (15.2)	25-88	58.7 (13.7)	25-90
Age at onset, y	48.3 (12.5)	36-61	46.0 (8.7)	38-65	.72	49.9 (14.3)	18-76	49.9 (13.9)	8-86
Disease duration, y	7.0 (2.6)	5-10	10.5 (7.2)	1-23	.43	6.9 (7.8)	0-33	8.7 (6.9)	0-52
Age at death, y	58.7 (15.6)	42-73 (3)	67.7 (14.4)	57-84 (3)	.50				
Clinical signs at onset									
Micrography, No.	0/3		1/7		>.99	10/41		107/370	
Rest tremor, No.	2/3		2/7		.5	18/43		218/371	
Bradykinesia, No.	2/3		6/7		>.99	21/43		226/372	
Asymmetry, No.	2/3		7/9		>.99	36/42		352/375	
Self-estimated levodopa improvement	36 (22)	17-60	53 (18)	40-60	.43	34 (19)	10-100	66 (19)	30-100
Clinical signs at examination									
Bradykinesia, No.	3/3		9/9			35/44		368/383	
Rigidity, No.	3/3		8/9		>.99	39/43		358/380	
Rest tremor, No.	2/2		6/9		>.99	30/47		295/377	
Urinary incontinence, No.	3/3		0/6		.01	11/40		25/367	
MMSE score ^c	15.0 (12.8)	6-24 (2)	27.8 (2.0)	26-30 (5)	.05	25 (7.0)	6-30 (18)	27.6 (4.0)	5-30 (212)
UPDRS score on ^d	44.5 (2.1)	43-46 (2)	11.7 (6.1)	5-17 (3)	.006	26.2 (16.2)	7-57 (12)	19.2 (13.5)	0-80 (199)
UPDRS score off ^d	51.2 (6.7)	47-56 (2)	42.7 (27.1)	5-86 (6)	.69	34.8 (14.9)	17-56 (10)	35.5 (19.0)	5-96 (130)
Daily dose of levodopa, mg	1000 (283)	800-1200 (2)	450 (397)	50-1100 (5)	.14	387 (364)	0-1200 (19)	577 (336); n=244	0-2300 (244)
Duration of treatment, y	NA		6.6 (3.4)	3-12 (5)		7.1 (6.9)	0.3-24 (19)	6.8 (6)	0.2-29 (304)
Dyskinesia	NA		4/5			7/21		154/325	
Fluctuation	NA		3/5			7/21		175/317	
Dystonia	NA		2/4			6/20		96/315	

Abbreviations: MMSE, Mini-Mental State Examination; NA, not available; PD, Parkinson disease; UPDRS, Unified Parkinson Disease Rating Scale.

^aComparisons of means and frequencies were calculated using SPSS software (SPSS Inc, Chicago, Illinois).

^bIndicates the number of patients with available data for the corresponding feature.

^cThe maximum score for the MMSE is 30 points.

^dThe maximum score for the UPDRS is 180. On and off indicate that the scale was rated with or without treatment with levodopa, respectively.

COMMENT

In addition to PD caused by SNCA multiplications, a very limited number of human genetic diseases have been reported to be caused by increased gene dosage, including autism spectrum disorder (OMIM 209850, caused by duplication or deletion of contactin 4 [CNTN4] (OMIM 607280)),¹⁷ Charcot-Marie Tooth disease type 1A (OMIM 118220, caused by duplication of the peripheral myelin protein 22 [OMIM 601097] locus),¹⁸ Pelizaeus-Merzbacher disease (OMIM 312080, caused by duplication of proteo-lipid protein 1 [OMIM 300401]),¹⁹ early-onset Alzheimer disease (OMIM 104760.0020, caused by duplication of the amyloid precursor protein [OMIM 104760] locus),²⁰ and hereditary pancreatitis (OMIM 167800, caused by triplication of the trypsinogen [OMIM 276000] locus).²¹ In this study, we found 4 of 264 families (1.5%) with typical ADPD that carried SNCA gene duplications. Our results are in accordance with a smaller study in an Asian population⁷ in which SNCA duplications were found in 2 of 113 families with ADPD (1.8%). Because SNCA triplications have been associated with dementia with Lewy bodies and PD dementia, we also screened a series of 22 patients with autosomal dominant atypical parkinsonism and found 1 family who carried an SNCA gene triplication (4.5%). In contrast, several other studies failed to identify SNCA rearrangements

in patients with PD or dementia with Lewy bodies, but the number of familial cases was limited.²²⁻²⁷ Our results suggest that SNCA duplications may be more frequent than point mutations in familial PD.

As anticipated from previous studies, duplications produced typical PD, whereas triplications resulted in an earlier onset and more aggressive disease with features reminiscent of dementia with Lewy bodies, indicating that the increase in the number of copies of SNCA has an effect on phenotype.⁶⁻¹¹ However, 2 recent studies reported SNCA duplications in patients with either dementia⁷ or variable symptoms resembling those of multiple system atrophy.⁶ The absence of dementia in our patients with SNCA duplications might be due to shorter disease durations or to ages at onset that were earlier than those previously reported (mean [SD], 46.0 [8.7] vs 52.0 [13.3] years).^{6,7,9} Conversely, the age at onset in our patients with an SNCA triplication was later (48.3 [12.5] years) than that of the Iowan (36.0 [10.5] years)¹¹ or Swedish American (31 years) patients.^{6,10} Comparison of patients carrying duplications and triplications of the SNCA gene showed no statistically significant differences in age at examination, age at onset, or disease duration. However, patients with triplications had shorter disease durations until death than duplication carriers. They had more severe dementia, were more severely affected, and had more frequent urinary incontinence than those with

duplications, underlining the dosage effect of *SNCA* multiplications.

The size of the rearrangements estimated by dosage of microsatellite markers was confirmed and refined by Affymetrix 250K SNP microarrays in all but one family who could not be analyzed because no more genomic DNA was available. Interestingly, our study shows that the size of the duplicated region and the number of genes it contains has no effect on phenotype. There are no additional signs or greater severity in family FPD-131 with at least 33 more duplicated genes than in families in which *SNCA* alone (FPD-437) or *SNCA* and *MMRN1* (FPD-410) were duplicated. Nishioka et al⁷ speculated that *MMRN1* overexpression may contribute to the dementia phenotype because one demented patient carried a genomic duplication containing both *SNCA* and *MMRN1* in their entirety, whereas the duplicated region in the patient without dementia contained all of *SNCA* but only part of *MMRN1*. Our results do not confirm this hypothesis because the patients carrying multiplications of both *SNCA* and *MMRN1* in our series did not have dementia. In addition, the *MMRN1* gene is not expressed in the brain.

Penetrance of *SNCA* multiplications is age dependent and might also be reduced, as suggested by previous studies.^{7,12} However, the genetic basis of this age-at-onset variability is not known. It is probably not related to the size of the multiplication because the 2 unaffected carriers belong to the family with the second largest duplication. We have also excluded the *LRRK2* G2019S mutation, which was absent in all of the families with *SNCA* multiplication, as a potential modifier.

The mutations found in the 5 families have different sizes, and the haplotypes in the rearranged region are not the same, indicating that all 5 mutations occurred independently. Furthermore, our haplotype analysis suggests that different mechanisms are involved in the generation of *SNCA* multiplications. For 3 duplications and the triplication, a single sequence was multiplied and transmitted in each family. This suggests an unequal intrachromosomal rearrangement after recombination between sister chromatids. In the 2 patients of family FPD-437, however, the microsatellite alleles on the 2 copies of the duplicated region were different, suggesting that the duplication resulted from a nonallelic homologous recombination between 2 homologous chromosomes. This mechanism is frequent in genomic disorders and is usually the consequence of nonallelic homologous recombination between low-copy repeats that flank unique genomic segments.²⁸ However, in our study, bioinformatics analyses of all of the multiplied regions containing *SNCA* revealed no large flanking low-copy repeats, but rather numerous repetitive elements interspersed at the breakpoints. Interspersed repetitive elements at breakpoints have been reported elsewhere and their presence has been postulated to be a frequent cause of deletions and duplications by nonallelic homologous recombination.²⁹⁻³¹ Interestingly, the most comprehensive study of genome-wide human segmental duplications found an enrichment of Alu repeats near or within breakpoints.³²

Our study indicates that the phenotype associated with *SNCA* multiplications correlates with the number of copies of the *SNCA* gene, but not with the number of copies

of other genes in the multiplied region, and provides the first insight into the mechanisms underlying *SNCA* multiplication. Because disorders resulting from genome rearrangements are thought to occur with equal frequency in all populations,³³ *SNCA* multiplications could account for approximately 2% of all ADPD cases.

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