4,000 ng/mL[8] but that of Japanese patients is around 500 (max 1,000) ng/mL (unpublished data; data from 200 Japanese PD patients). Race, amount of protein intake, and physique may explain the difference in the effective levodopa dose between Western countries and Japan. The above data indicate that our patients were not undertreated with anti-PD drugs. In fact, our patients, like other Japanese patients with PD, developed treatment-related adverse effects during maintenance therapy using levodopa with or without other drugs. Nevertheless, further studies are necessary to evaluate ZNS in patients with PD treated with anti-PD drugs at doses commonly used in Western countries.

We started the study with a run-in period of single-blind treatment with placebo to minimize placebo effects. To the best of our knowledge, this is the most rigorous study design used to date for the evaluation of anti-Parkinson effects. Our study design may explain the lower response rate in the ZNS groups (although still significantly higher than placebo in the 25-mg and 50-mg study groups) than that of previous reports for pramipexole.[9]

Although there was a higher incidence of adverse events in the 100-mg group than in the other treatment groups, the incidence of hallucination and dyskinesia, which are typically of concern with anti-Parkinson drugs, was the same across all treatment groups, indicating that a once-daily dose of 25 to 100 mg of ZNS is well tolerated.

Although the present study was only of 12 weeks' duration, our preliminary data showed that the benefits observed at 12 weeks were maintained for more than 1 year in all 17 patients in a study on the long-term effects of ZNS on PD. Another study that was designed to assess the long-term (up to 1 year) effects of ZNS on PD ($n = 100$) also showed that 12-week course of ZNS improved parkinsonian symptoms and that such effects were maintained for up to 1 year (manuscript in preparation).

It is notable that the typical dose of ZNS is 300 to 600 mg/day for epilepsy, but a significant improvement in motor symptoms was noted in our patients with PD with only 50 mg/day of ZNS. This suggests that the mechanism of action of ZNS in PD may be different from those in epilepsy. In this regard, ZNS has multiple mechanisms of action. The major effect of ZNS in epilepsy is modification of neuronal firing at high frequency through enhancement of sodium channel inactivation and reduction of T-type calcium current.[10-13] ZNS has no affinity to γ-aminobutyric acid (GABA) type A receptor or glutamate receptors[11] but is known to increase GABA[6] and glutamate[7] release. In the dopaminergic system, therapeutic doses of ZNS (20 and 50 mg/kg) increase intracellular and extracellular dopamine levels in the rat striatum.[14-16] Conversely, supratherapeutic doses of ZNS reduce intracellular dopamine. Thus, ZNS has biphasic effects on the dopamine system. We reported previously that at therapeutic levels, ZNS increased dopamine synthesis by increasing tyrosine hydroxylase (TH) activity and TH messenger RNA.[14] ZNS also affects MAO-B activity. The IC_{50} (50% inhibitory concentration) value of MAO-B in liver microsomal fraction was 600 μM, and that in striatal membrane fraction was 28 μM.[14,17] These data suggest ZNS inhibits striatal MAO-B activity but not peripheral MAO-B activity, and therefore ZNS may have little effect on peripheral MAO-B inhibition of functions such as blood pressure.

Zonisamide has no affinity to dopamine receptors (D1–D5) or dopamine transporter. ZNS also has no direct effects on glutamate receptors, adenosine receptors, or serotonin receptors, which have been suggested as possible sites of action for anti-PD drugs, other than the dopaminergic system.[14] We proposed previously that activation of dopamine synthesis and moderate inhibition of MAO-B are the main mechanisms that mediate the effects of ZNS in PD.[14] However, the present finding of lack of change in the efficacy of ZNS when coadministered with an MAO-B inhibitor suggests that MAO-B inhibition is not a main factor. We consider that the primary mechanism of action of ZNS in PD is to increase dopamine synthesis. Whether sodium channel inactivation or T-type calcium channel inhibition is involved in ZNS effects has not been elucidated yet. Further investigation is needed to clarify the mechanism of the beneficial actions of ZNS on PD.

Appendix

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Clinical Heterogeneity of α-Synuclein Gene Duplication in Parkinson's Disease

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Objective: **Recently, genomic multiplications of α-synuclein gene (*SNCA*) have been reported to cause hereditary early-onset parkinsonism. The objective of this study was to assess the frequency of SNCA multiplications among autosomal dominant hereditary Parkinson's disease (ADPD).** ***Methods:*** **We screened 113 ADPD probands and 200 sporadic PD cases by quantitative polymerase chain reaction and confirmed *SNCA* multiplications by flurorescence in situ hybridization (FISH) and comparative genomic hybridization array.** ***Results:*** **Two families (two patients from Family A and one from Family B) with SNCA duplication were identified among ADPD patients. Even though they had the same *SNCA* duplication, one patient had dementia. Because there was exactly the same difference between the regions originated from each patient, the finding suggests that the phenotype of *SNCA* multiplication may be also influenced by the range of duplication region. We also detected asymptomatic carriers in the families of both patients. Interestingly, the penetrance ratio was 33.3% (2/6) in one kindred, indicating that the ratio was very much lower than expected.** ***Interpretation:*** **These two newly identified Japanese patients with *SNCA* duplication and the five previously identified American and European families with *SNCA* triplication or duplication mutations indicate that the incidence of *SNCA* multiplication may be more frequent than previously estimated.**

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Parkinson's disease (PD) is the second most common neurodegenerative disorder next to Alzheimer's disease (AD). Although the exact cause for PD remains to be elucidated, genetic factors could contribute to the pathogenesis of PD. Indeed, six causative genes and four chromosomal loci for familial PD (FPD) have been identified.[1–13] *α-Synuclein*, *UCH-L1*, and *LRRK2* have been identified as causative genes for autosomal dominant forms of FPD (ADPD), whereas *parkin*, *PINK1*, and *DJ-1* have been identified as causative genes for autosomal recessive forms of FPD (ARPD).[1,10,14] The presence of several causative genes and loci for FPD indicates that the pathogenic mechanisms of sporadic PD are also multifactorial. Studies of FPD are important as they enhance our understanding of nigral neuronal death. Furthermore, it has been proposed that the gene products for FPD are components of common pathways in sporadic PD. As testament, missense mutations such as A30P,[15] E46K,[16] and A53T,[9] in the N-terminal of α-synuclein gene (*SNCA*) have been linked to a rare form of FPD, and α-synuclein subsequently was confirmed to be a major component of Lewy bodies (LBs) and Lewy neurites, the pathological hallmark of sporadic PD and dementia with LBs (DLB).[17] Based on large population-based studies, missense mutations of *SNCA* are infrequent.[18] In particular, the SNCA A53T mutations identified in patients with FPD originate from a single founder. To date, SNCA A30P and E46K mutations have been found in only one family each, suggesting that missense mutations are a very rare cause of parkinsonism. Recently, *SNCA* multiplications in FPD haves been re-

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ported in two families with genomic triplication and in three families with duplications.[11–13,19] These findings suggest that overproduction of α-synuclein is one of the most important factors in FPD. In general, unequal intrachromosomal crossovers that result from misalignment of two homologous flanking sequences may account for genomic multiplications as well as deletions. The *SNCA* multiplications mutations, triplications, and duplications found in five unrelated patients probands with FPD are de novo within each kindred.[11–13,19] Affected individuals within the Iowa kindred, with *SNCA* genomic triplication, have fulminant, early-onset disease with a phenotype ranging clinically and pathologically from PD to diffuse LB disease (DLBD).[20] In contrast, *SNCA* duplication families have later onset disease and a longer duration to death, and neither cognitive decline nor dementia are prominent. Therefore, overproduction of wild-type α-synuclein (*SNCA*) may result in phenotypes of PD, PD with dementia (PDD), and DLBD, suggesting that regulation of α-synuclein protein levels is central to the cause of these phenotypes. In summary, the phenotype may be dependent on copy numbers of *SNCA*.

In this study, to gain further insight into the role of this multiplication, we assessed a series of 113 PD patients with autosomal dominant mode of inheritance and 200 sporadic PD patients for multiplication at this locus.

Subjects and Methods

Patients

This study consisted of 113 patients with ADPD and 200 patients with sporadic PD. Diagnosis of PD was adopted by the participating neurologists and the diagnosis was established based on the United Kingdom Parkinson's Disease Society Brain Bank criteria.[21] The mean age at onset of the 56 male and 57 female index patients with ADPD was 66.0 ± 9.5 (±SD), and that of the 81 male and 119 female patients with sporadic PD was 64.7 ± 10.0 (±SD). All patients were of Japanese origin. The study was approved by the ethics review committee of Juntendo University. Blood samples for genetic analysis were collected after obtaining informed consent from each patient and 17 unaffected relatives. None had mutations in *parkin*, *PINK1*, or *DJ-1*. We could not detect heterozygous exon deletions of such recessive genes by quantitative analysis in the patients studied. In addition, none had mutations in exon 41 in *LRRK2*.

Gene Dosage Analysis for SNCA

DNA was prepared using standard methods. The mutation screening was performed as described previously.[22] Semiquantitative multiplex polymerase chain reaction (PCR) of genomic DNA samples was performed using a real-time PCR method to detect the dosage of *SNCA* (ABI Prism 7700 sequence detector; Applied Biosystems, Foster City, CA). As the first step, we targeted exon 3 of *SNCA* to screen the gene dosage of *SNCA*. β-*Globin* gene was amplified as an endogenous reference. In addition, we used a DNA sample from the Iowa family (patients had triplication of *SNCA*) as a positive control. The primer and TaqMan MGB probe sequences used in this study are described in Table 1. PCR was conformed with PCR universal master mix using 25ng of genomic DNA, 900nM primers, and 250nM probes (β-globin is 50–200nM) in a total reaction volume of 50μL. PCR cycling conditions were 95°C for 10 minutes, 95°C for 15 seconds, and 60°C for 1 minute (40 cycles). Values between 0.4 and 0.6 were considered as heterozygous deletion, between 0.8 and 1.2 as normal, between 1.3 and 1.7 as heterozygous duplication, and greater than 1.8 as triplication.

In the second step, we performed semiquantitative analysis on exons 1/2, 4, 6, and 7 for the patients found to carry multiplication of this gene in the first step. All the sequences of this gene are shown in Table 1.

Fluorescence In Situ Hybridization Analysis

We used two-color standard fluorescence in situ hybridization (FISH) and prophase FISH for metaphase and interphase. FISH analyses were performed as described previously,[23] using a BAC located around the region of interest. The location of each bacterial artificial chromosome (BAC) was archived by the database of UCSC (http://genome.ucsc.edu) or NCBI (http://www.ncbi.nlm.nih.gov). Two BAC contigs representing the region at 4q21-22. BACs RP11-17p8 and RP11-61407 were used as probes. BAC RP11-17p8 locates at site of centromere of chromosome 4, and BAC RP11-61407 locates at site of telomere of the same chromosome. PR11-61407 contains *SNCA*, suggesting that the signal of this clone shows the copy numbers of *SNCA*. The distance between the two BAC clones was approximately 1.4Mb. Probes were labeled with biotin-16-dUTP or digoxigenin-11-dUTP by nick-translation (Roche Diagnostics, Tokyo, Japan). The copy number of the region was assessed according to the hybridization patterns observed on both metaphase and interphase chromosomes. We established Epstein–Barr virus (EBV)–transformed lymphoblastoid cell line as described previously.[24]

Multiplication (duplication) Region Using Comparative Genomic Hybridization Array and Gene Dosage Technique

The triplication region in Iowa family is between 1.61 and 2.04Mb and contains 17 annotated or putative genes. A recently constructed high-density comparative genomic hybridization (CGH) array, designated MCG Whole Genome Array-4500,[25] which contains 4532 BAC/P1-artificial chromosome (PAC) clones covering the entire genome at intervals of approximately 0.7Mb, was used for CGH array analysis. This array is suitable for detecting the size of the multiplication if the size is greater than 0.7Mb. Hybridizations were performed as described previously with minor modifications.[26,27] In brief, test and reference genomic DNAs from the patient's lymphoblastoid cells and normal lymphocytes, respectively, were labeled with Cy3- and Cy5-dCTP (Amersham Biosciences, Tokyo), respectively, precipitated together with ethanol in the presence of Cot-1 DNA, redissolved in a hybridization mix (50% formamide, 10% dextran sulfate, 2 × standard saline citrate [SSC], and 4% sodium dodecyl sulfate [SDS], pH 7.0),

Table 1. Sequences of Primer and TaqMan Probes Used in the This Study

		Forward Primer	Reverse Primer
β-globin	—	5′-TGGGCAACCCTAAGGTGAAG-3′	5′-GTGAGCCAGGCCATCACTAAA-3′
ABCG2	Exon 1	5′-GGAAGGTCCGGGTGACTCA-3′	5′-GGAGGCAGCGCTTTAACAAT-3′
—	Exon 2	5′-GTGTCACAAGGAAACACCAATGG-3′	5′-AGCTCCTTCAGTAAATGCCTTCAG-3′
DFKZ	Exon 7	5′-CTGGACCACTTACTGGTGAAAGC-3′	5′-CACTGTGCCTGGCCAAATT-3′
FAM13A1	Exon 12	5′-GAAGAGGACCTAACTCCCAGGAT-3′	5′-TTCTCAAGTTGGGAACCAAAACTCT-3′
LOC345278	Exon 8	5′-GTTGGCTGGGCCAATCTCT-3′	5′-TGGTCTTAGCTGAAGGCCAGTT-3′
SNCA	Exon s1/2	5′-CCTTCAAGCCTTCTGCCTTTC-3′	5′-CGAATGGCCACTCCCAGTT-3′
—	Exon 3	5′-TTCCAGTGTGGTGTAAAGAAATTCAT-3′	5′-CCTTGGCCTTTGAAAGTCCTT-3′
—	Exon 4	5′-CAGCAATTTAAGGCTAGCTTGAGACT-3′	5′-CCACTCCCTCCTTGGTTTTG-3′
—	Exon 6	5′-TATGCCTGTGGATCCTGACAAT-3′	5′-TCAGCTTGGACTCCTACCTCAGA-3′
—	Exon 7	5′-TCTTTGCTCCCAGTTTCTTGAGA-3′	5′-TGGAACTGAGCACTTGTACAGGAT-3′
MMRN1	Exon 1	5′-ATCAAACTCTCACATCCCAC-3′	5′-CACCTGCTGAGGGTGTGAGA-3′
—	Exon 5	5′-CAGGCAATGAAACTGACTCTTCTG-3′	5′-CTTCTAGGGAGGAGTAAGTGTTCCT-3′
—	Exon 6	5′-GTTTCAATAGCAGCCCAGCAAAA-3′	5′-CAGTCAAAGTGGGCCGATTCT-3′
—	Exon 8	5′-GCTTCATATACCCCAAGAACTGGAA-3′	5′-GCACTAAATGACTCGATGGTGTACT-3′
KIAA1680	Exon 1	5′-TTAAATAACGCAGCTGGACTCTGT-3′	5′-TTAAATAACGCAGCTGGACTCTGT-3′
—	Exon 2	5′-GGCCACAATGATTCTACCTCTCA-3′	5′-CCGTAAGTTCTGTTGTTGTCTTTGT-3′
—	Exon 3	5′-AGCTCAGGTAGCACAGGTAAACG-3′	5′-TGGTGGAAGCTAATGGAAGGA-3′
—	Exon 4	5′-CCATTTCGTGAAGGAAGATTTATAGAG-3′	5′-TCCCTGCAGTGCCTTCTGA-3′
—		MGB probe	
β-globin	—	5′-CTCATGGCAAGAAAGTGCTCGGTGC-3′	
ABCG2	Exon 1	5′-CCCAACATTTACATCCTT-3′	
—	Exon 2	5′-CCGCGACAGCTTCCAA-3′	
DFKZ	Exon 7	5′-ACCATGCAAAAGAAAT-3′	
FAM13A1	Exon 12	5′-AAGCAACACACTCCCC-3′	
LOC345278	Exon 8	5′-CAGAAGCTGACTCTCA -3′	
SNCA	Exons 1/2	5′-ACCCTCGTGAGCGGA-3′	
—	Exon 3	5′-AGCCATGGATGTATTC-3′	
—	Exon 4	5′-TGTCTTGAATTTGTTTTTGTAGGC-3′	
—	Exon 6	5′-AGGCTTATGAAATGCC-3′	
—	Exon 7	5′-TGCTGACAGATGTTC-3′	
MMRN1	Exon 1	5′-ACTTGACCACTCCTTCTGCTTTCT-3′	
—	Exon 5	5′-CACAGTCAAAGAAATATTG-3′	
—	Exon 6	5′-CTTGCACCAAAACAAAC-3′	
—	Exon 8	5′-TCCAAGATACGGAATTCTA-3′	
KIAA1680	Exon 1	5′-TCCCCTTCTCGGCTGTTG-3′	
—	Exon 2	5′-ATGTCCCTCAATTCTG-3′	
—	Exon 3	5′-AGGAGCATATTCCG-3′	
—	Exon 4	5′-AGACTGCGATCCTC-3′	

and denatured at 75°C for 8 minutes. After 40-minute preincubation at 42°C, the mixture was applied to array slides and incubated at 50°C for 10 minutes, 46°C for 10 minutes, and 43°C for 60 hours in a hybridization machine, GeneTAC (Harvard Bioscience, Holliston, MA). After hybridization, the slides were washed once in a solution of 50% formamide, 2 × SSC (pH 7.0) for 10 minutes at 50°C and 1 × SSC for 10 minutes at 42°C, respectively, and then scanned with a GenePix 4000B (Axon Instruments, Foster City, CA). The acquired images were analyzed with GenePix Pro 4.1 imaging software (Axon Instruments). Fluorescence ratios were normalized so that the mean of the middle third of $\log_2$ ratios across the array was zero. The average values for each clone were within the thresholds of 0.2 and −0.2 (log2ratio), and the mean ± 2 SD values of all clones were within the range of 0.4 and −0.4 (log2ratio). The thresholds for copy number gain and loss were set at log2 ratios of 0.4 and −0.4, respectively.

We picked up the locus region between *ABCG* and *KIAA1680* of approximately 1.6 to 2.0 Mb. To identify the region of duplication spanning *SNCA*, we performed semiquantitative PCR on target genes including *ABCG, DFKZ, FAM13A1, LOC345278, MMRN*, and *KIAA1680* using the same methods. The sequences of all primer and probe sets are shown in Table 1.

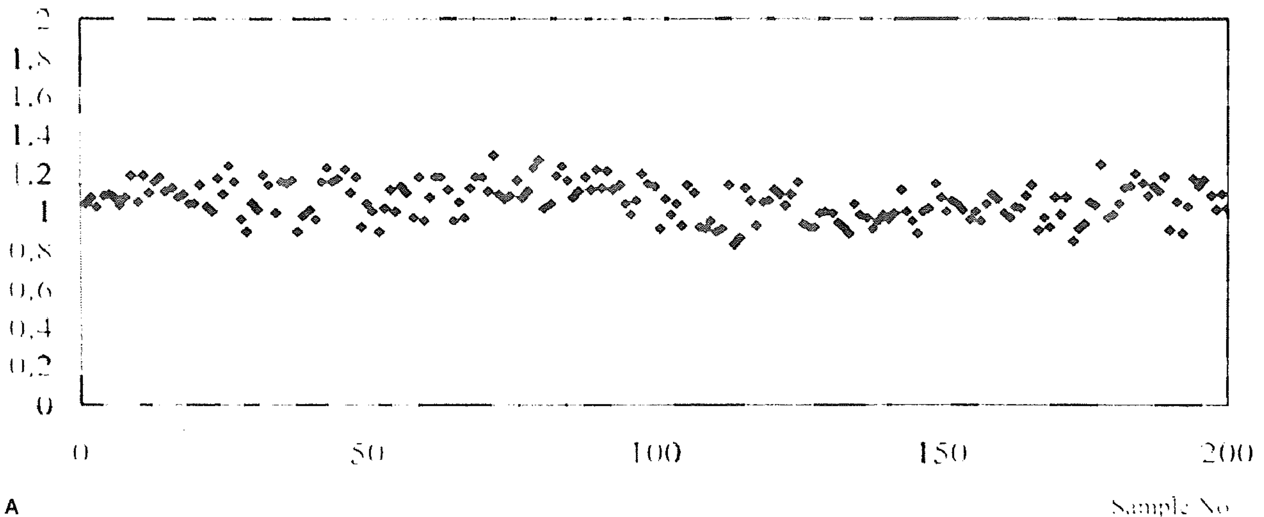
Haplotype Analysis

To determine whether the same haplotype was shared between our probands with *SNCA* multiplication, we performed haplotype analysis in patients with *SNCA* duplication from unrelated families. We used four microsatellite markers including *D4S2361, D4S2505E* (located within *SNCA*), *D4S2380, D4S1647*, and *D4S421*.

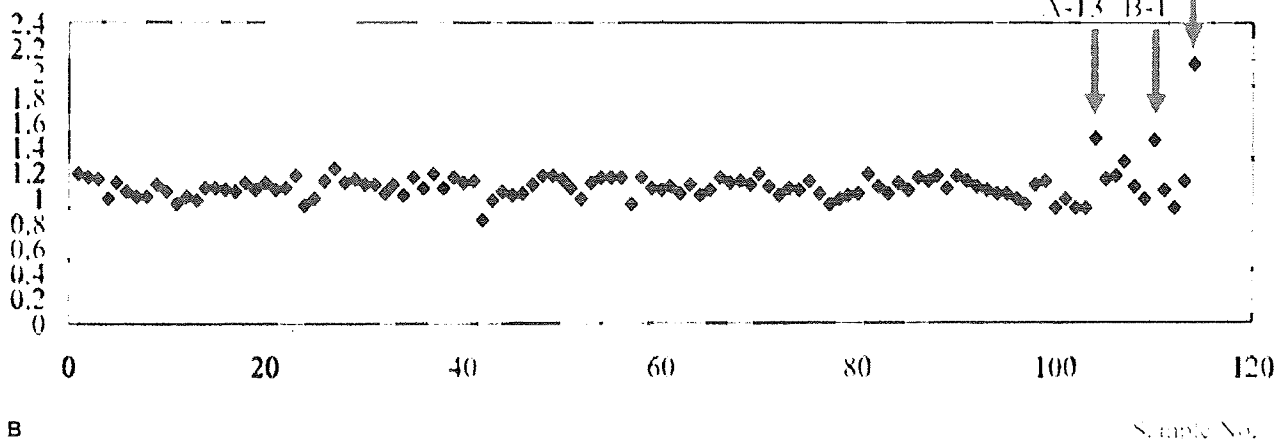
Results

Gene Dosage Analysis for α-Synuclein

Using semiquantitative PCR to detect gene dosage, we did not find patients harboring *SNCA* multiplication



A



B

Fig 1. The ratio of α-synuclein exon 3, used as a target gene, to β-globin, used as a reference gene, as determined by semiquantitative real-time polymerase chain reaction in: (A) 200 patients with sporadic PD (the ratio ranged from 0.8 to 1.3, suggesting that single SNCA *copy exists in one allele, and (B) 113 patients with autosomal dominant hereditary Parkinson's disease. Note the two cases of duplication ratio (the ratio is 1.46 in one patient and 1.48 in the other), and the single Iowa family triplication case with a ratio of 2.07.*

among 200 sporadic cases (Fig 1A) but detected two index patients (A-13 and B-1) with potential *SNCA* duplications among 113 autosomal dominant pedigrees using exon 3 of *SNCA* (Fig 1B). To confirm the entire region of the α-synuclein gene was multiplied, we performed the exon dosage analysis including exons 1/2, 4, 6, and 7. We confirmed duplication of this gene in two patients. Thus, we were able to confirm that two families (Families A and B) were ADPD with *SNCA* duplication. In Family A, two patients with duplication had typical PD whereas five carriers were asymptomatic (Fig 2A). In Family B, one patient had duplication of the *SNCA* gene; two members were carriers (see Fig 2B).

FISH analysis also confirmed the *SNCA* duplication in the two index patients (Fig 3A, B). Figure 3 shows

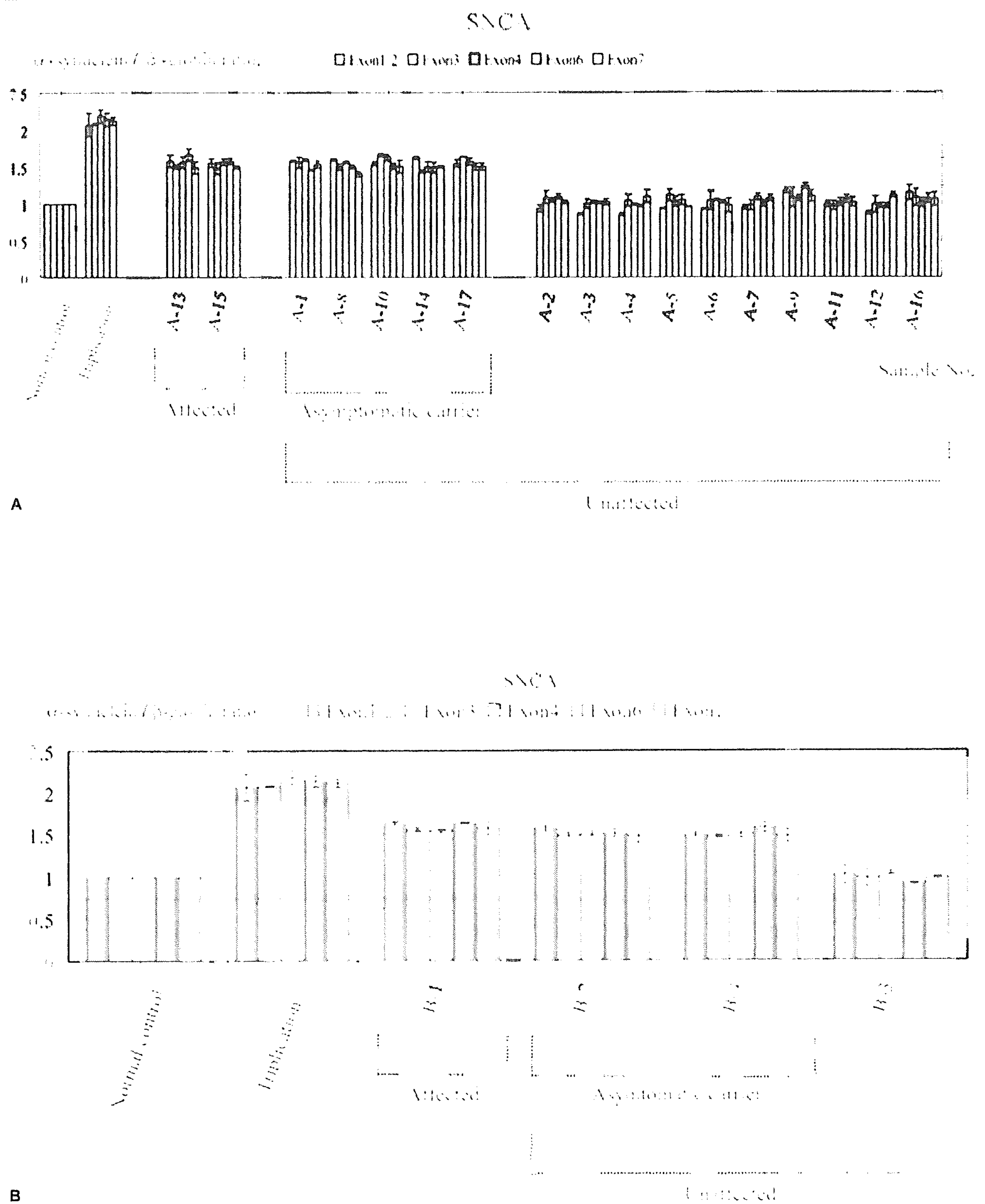


Fig 2. Results of screening for SNCA *multiplications for exons 1 to 7 in Family A (A). We detected two patients with* SNCA *duplication and five asymptomatic carriers in this family (a penetrance ratio of 33.3%) and (B) Family B. We detected three patients with* SNCA *duplication in four family members.*

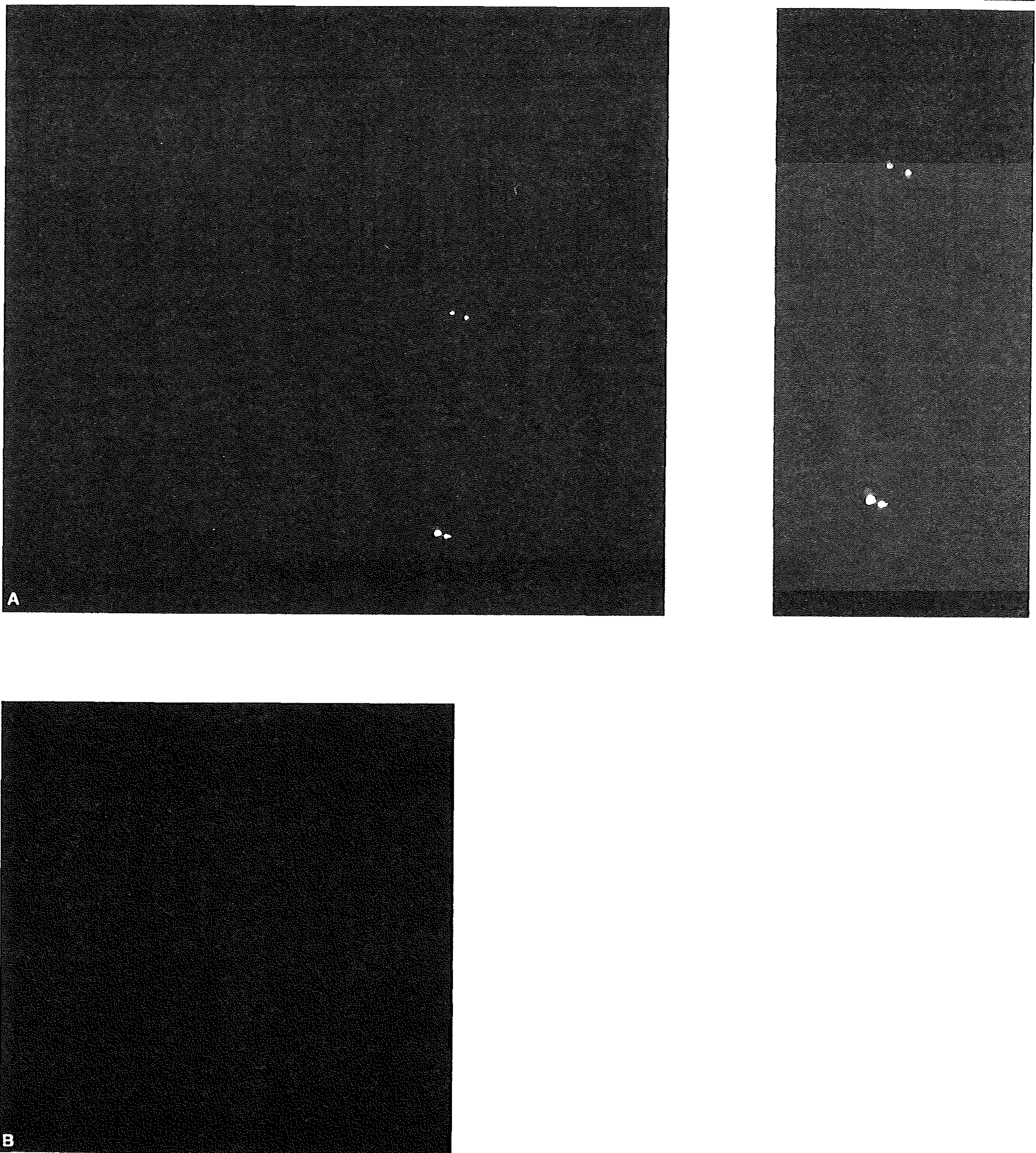


Fig 3. (A) Schematic representation of fluorescence in situ hybridization assay of metaphase chromosomes from Epstein–Barr virus (EBV)–transformed lymphocytes derived from Patients A-13 and B-1. We used BACs RP11-17p8 for normal control sample (shown in green and located 1.4Mb centromeric to SNCA*, left panel) and RP11-61407, which included the* SNCA *shown in red on chromosome region 4q21-22 (right panel). These pictures show clearly disproportional segregations compared with the normal control. (B) Standard one-color FISH of the interphase, using BACs RP11-61407. Note the two disproportional signals.*

the representative results of FISH analysis of interphase and metaphase chromosomes from EBV-transformed lymphocytes derived from Patients A-13 and B-1. We detected tight apposition of the metaphase chromatids compared with signals of BAC RP11-17P8 located 1.4Mb centromeric to *SNCA*. The intensity of the sig-

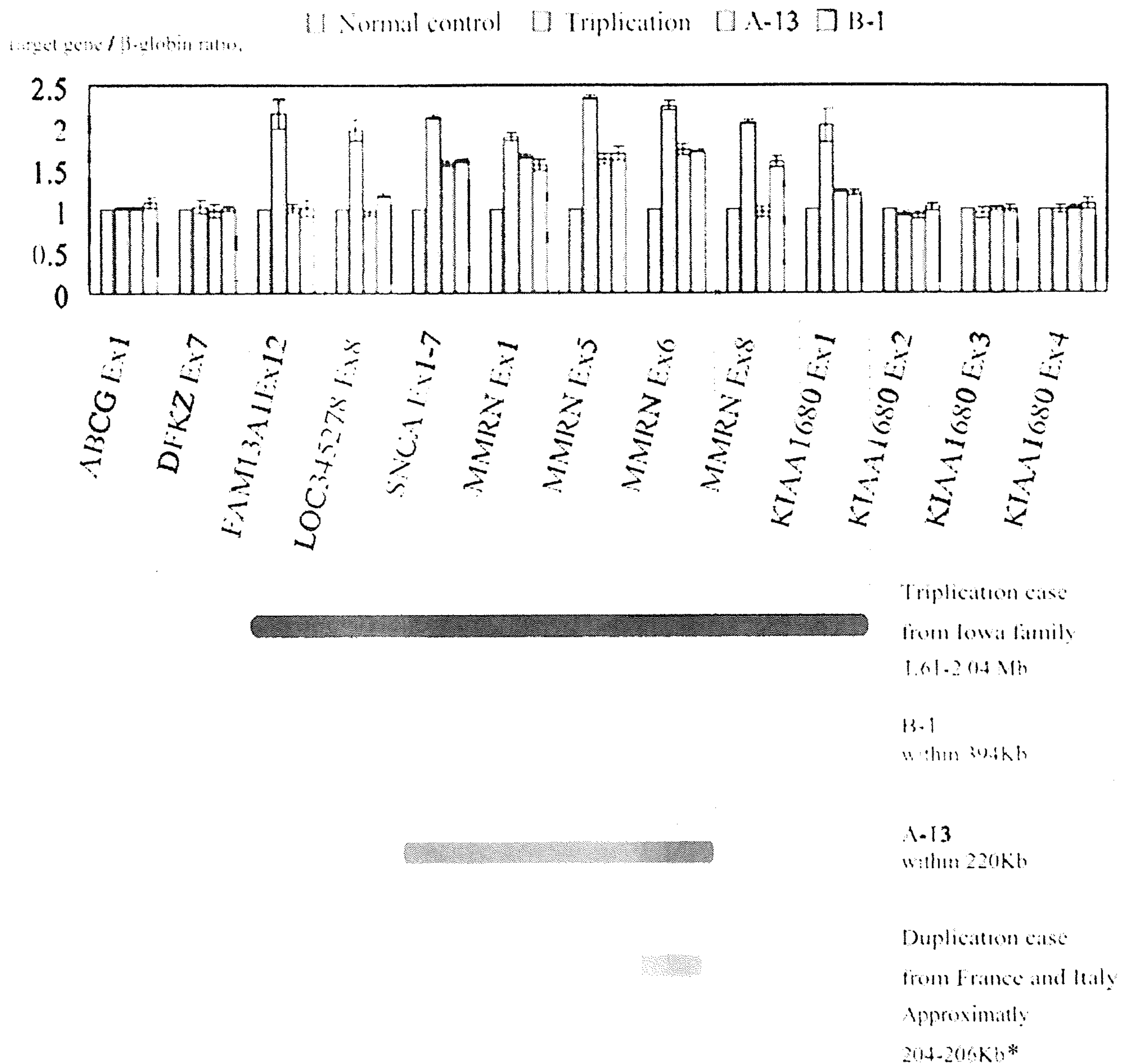


Fig 4. Identification of the region of SNCA *duplication between* ABCG *to* KIAA1680 *by using real-time semiquantitative polymerase chain reaction method. The different duplication region appears on* MMRN1 *Exon 8. *Duplication case as reported in Ibanez et al.*[13]

nal suggests *SNCA* duplication in these two patients. When considered together with the results of gene dosage analysis, we were able to confirm *SNCA* duplication. We did not observe two separate signals between BACs RP11-17P8 and PR11-61407, suggesting that the size of the duplication region is less than 1.4Mb. CGH array analysis showed that the specific elevation ratio could not be detected because the *SNCA* region could not be directly included in BAC probes used in MCG Whole Genome Array-4500. However, this BAC-based array contains BACs RP11-49M7 and RP11-17p8 that are close to 5′ or 3′ sites of *SNCA*, respectively. Alternatively, this finding indicates that the *SNCA* duplication region is less than 0.7Mb based on information archived by the database of UCSC (http://genome.ucsc.edu) and NCBI (http://www.ncbi.nlm.nih.gov). Although MCG Whole Genome Array-4500 covers the entire genome, no specific multiplication or deletions existed in other regions apart from 4q21-22. Identification of the *SNCA* duplication region was carefully assessed by gene dosage analysis for flanking genes around *SNCA* (Fig 4). The length of *SNCA* duplication of Patient A-13 spanned all of *SNCA* and part of *MMRN1* such as exons 1 to 6. In

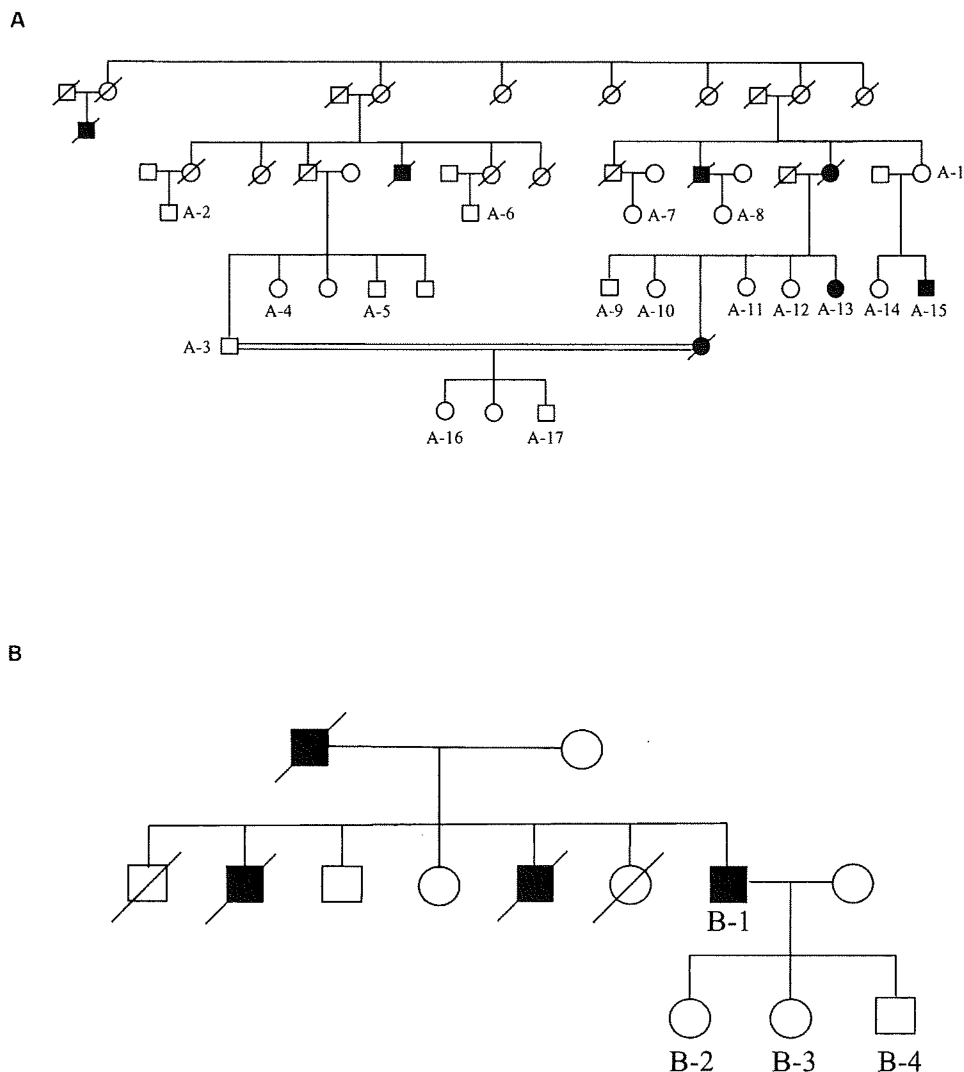


Fig 5. (A) Pedigrees of Patient A-1 with Parkinson's disease (PD) showing four generations. Black boxes represent affected patients. Symbols with numbers represent family members who were examined clinically by neurologists and from whom blood samples were collected. In 17 members, two patients were affected and five members (A-1, A-8, A-10, A-14, A-17) were carriers. Among seven carriers with SNCA *duplication, the ages of all carriers except for A-17 were beyond the mean age at onset of patients with* SNCA *duplication. Thus, the penetrance ratio was 33.3% (two patients/six asymptomatic carriers). (B) Pedigree of Patient B-1 with PD showing three generations. Symbols are as for Figure 6A. In four members, one patient was affected, and two members were carriers.*

contrast, the duplication region of Patient B-1 spanned all of *SNCA* and *MMRN1*. In addition, the regions of both patients did not span *LOC345278* and in Patient B-1, no duplication of *KIAA1680* was observed. Thus, the length of the duplication of Patient A-13 was shorter than that of Patient B-1, suggesting that the different lengths of the duplications differ by approximately 100 to 200kb. Furthermore, these two families have different allele sizes in microsatellite markers, suggesting that SNCA duplication is also de novo (data not shown). Clinical data, including the results of neuroimaging such as magnetic resonance imaging (MRI) and single-photon emission computed tomography (SPECT) and [^{123}I] meta-iodobenzylguanidine (MIBG) myocardial scintigraphy, are described below.

Family A

We collected DNA samples from 17 members of this family, including three affected and 14 unaffected members (Fig 5A). Among the three affected members, one patient (A-2) had no *SNCA* duplication. In addition, the age at onset of parkinsonism was 74 years. Moreover, L-dopa responsiveness was not excellent. Although MRI examination was not available, we considered that the cause of PD in this patient was not duplication but rather vascular parkinsonism based on

neurological findings. The mean age at onset of the disease was 43 years. The parents of A-16 and A-17 were close relatives. Five asymptomatic carriers were recognized by genomic analysis. No parkinsonism was observed in these asymptomatic carriers based on clinical neurological examination by two expert neurologists (K.N. and N.H.). The youngest age at onset was 38 years including the deceased patient (50 years old at onset). Thus, age 43 years was the cutoff age in this family. Considering this point, the penetrance ratio was 33.3% (2/6).

Patient A-13

The age of onset was 48 years. The initial symptom in Patient A-13 was rigidity and bradykinesia. She responded well to L-dopa. Six years after commencement of treatment with L-dopa, she developed drug-induced dyskinesia, which subsequently showed marked resolution. No tremor at rest has yet been noted. During the day, clinical assessment indicated Hohen and Yahr stage III. No dementia has developed yet and she has no symptoms related to autonomic nervous system dysfunction. Brain MRI study showed no abnormal mass or ischemic changes (Fig 6A) and ^{123}I-IMP SPECT study showed no evidence of hypoperfusion. However, the H/M ratio of MIBG myocardial scintigraphy was less than that of the normal control (A-13; early: 1.4, late: 1.24; see Fig 6D, E).

Patient A-15

The age at onset was 38 years. This patient was the cousin of Patient A-13. The initial symptom was gait disturbance with frequent falls. Tremor and autonomic nervous dysfunction were not seen. He was diagnosed with depression during the course of the disease, but neither dementia nor cognitive deterioration was prominent. The clinical course of this patient was similar to that of Patient A-13. Although this patient responded to L-dopa, he showed excellent response to anticholinergic agents such as trihexyphenidyl hydrochloride rather than L-dopa. In addition, the patient developed psychosis at 43 years of age.

Family B

DNA samples were collected from four members of Family B (see Fig 5B). Among the two generations, the number of affected member was four including three deceased members, and the unaffected members were three including two carriers with *SNCA* duplication. The age of asymptomatic carriers (B-2, B-3, and B-4) was younger than 35 years at the time of collection of DNA samples. Thus, it is difficult to speculate whether these carriers will develop PD in the future.

Patient B-1

The age at onset was 47 years. In the early stage, he responded to L-dopa; however, at 58 years of age, the disease was evaluated as stage III. Moreover, the gait disturbance and bradykinesia worsened and he suffered from cognitive dysfunction a few years later. Since 61 years of age, he has found it difficult to communicate with others and started gradually to develop abnormal behavior. Mini-Mental State Examination score was 17/30 at 61 years of age. At 62 years, his gait disturbance and hallucination worsened. At 64 years, he spent most of the day on the bed and required tracheostomy because of repeated episodes of aspiration pneumonia. Brain MRI showed moderate dilation of Sylvian fissure and atrophic changes in the temporal lobe on both sides. There was no evidence of ischemic changes or abnormal mass (see Fig 6B). A 99m-Tc-ECD SPECT study showed hypoperfusion predominantly on both frontotemporal lobes (see Fig 6C). The H/M ratio of MIBG myocardial scintigraphy was reduced (B-1; early: 1.40, late: 1.24).

Subject B-4

Subject B-4 was mentally retarded and had autism and generalized seizure. Since 1 year of age, he could not speak and was diagnosed with mental retardation by a pediatrician. At 12 years of age, he started to speak a few words and was sometimes observed to have sudden outburst of rage. At 15 years, he developed generalized seizure. EEG showed spiking waves predominantly localized to the right frontal lobe. Brain computed tomography scan showed no abnormal densities or other signs. No parkinsonism has been noted so far.

Table 2 summarizes the clinical features of these cases, including the results of neuroimaging and MIBG scintigraphy.

Discussion

Several recent studies suggest *SNCA* multiplications are a rare cause of PD, PDD, and DLBD.[22,28,29] In this study, we detected *SNCA* duplication in PD patients from 2 of 113 unrelated Japanese families with autosomal dominant parkinsonism. Thus, the incidence of *SNCA* multiplication may be more frequent than previously estimated. To our knowledge, the Iowa family and a single family of Swedish-American descent have been reported previously to have *SNCA* triplication.[11,19] In addition, two French families and one Italian family with *SNCA* duplication have been reported.[12,13] Taken together with this study, a total of seven families with *SNCA* multiplication, including triple and double *SNCA* copies, have been reported worldwide.

For all patients with *SNCA* duplication reported here, including patients of Family A, the phenotype was indistinguishable from idiopathic PD and no other

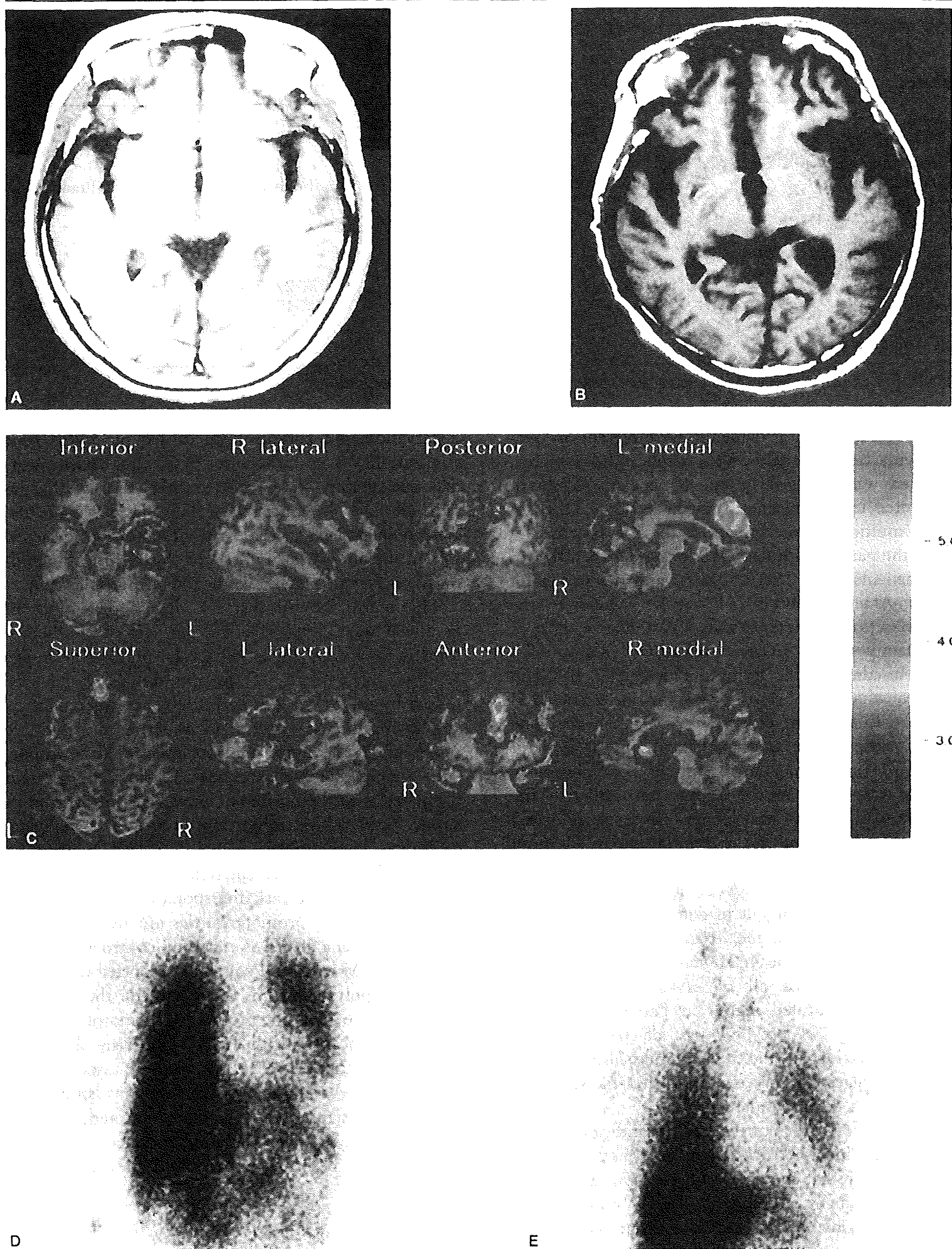


Fig 6. (A) Brain magnetic resonance imaging (MRI) T1 wedge study of Patient A-13. No abnormal masses or ischemic changes were evident. (B) Brain MRI T1 wedge study of Patient B-1. Note the dilation of Sylvian fissure and atrophic changes in both temporal lobes. (C) ^{123}I-IMP SPECT study of Patient B-1. Note the hypoperfusion of both frontotemporal lobes and medial-occipital lobes. (D, E) [^{123}I]meta-iodobenzylguanidine (MIBG) myocardial scintigraphy (D; early, E; late) of Patient A-13. The H/M ratio was reduced in this patient.

Table 2. Clinical Features of Four Affected Patients in Two Unrelated Pedigrees

	A Family			B Family
Feature	A-13	A-15	A-2	B-1
Age (yr)	57	43	77	65
Age at onset (yr)	48	38	74	47
Disease duration (yr)	10	6	4	19
Initial symptom	Rigidity	Rigidity	Bradykinesia	Bradykinesia
Bradykinesia	+	+++	++	+++
Rigidity	+++	+++	++	+++
Resting tremor	−	−	−	−
Postural instability	−	+	+	−
UPDRS	10/108	32/108	27/108	−
MMSE	30/30	30/30	17/30	17/30
L-Dopa response	+++	+	−	+
SNCA duplication	+	+	−	+

UPDRS = Unified Parkinson's disease rating scale; MMSE = Mini-Mental Status Examination.

clinical features such as dementia were present, in contrast with families with *SNCA* triplication. Notably, dementia was observed in one patient of Family B. Therefore, it is important to screen PDD or DLB for *SNCA* multiplications. However, the age of onset of PD in the patient with dementia was older than that of Iowa patients (36.0 ± 10.5 years) and the patient of Swedish-American family (31 years).[19] Moreover, the age at onset of Japanese patients was similar to those of other families with *SNCA* duplication (48.4 ± 15.0 years). In addition, the asymptomatic carrier, B-2, had epilepsy, which has been reported in one French PD patient.[13] In addition, autism was observed in the same patient, although no clear parkinsonism was evident. Patient B-1 had dementia, in contrast with previously reported cases with *SNCA* duplication, although the duration of the disease was longer (18 years) compared with reported cases of *SNCA* duplication. In addition, dementia only appeared after 14 years of diagnosis of parkinsonism. Therefore, *SNCA* duplication may be a risk factor for development of dementia.

Within each kindred the SNCA multiplication is a de novo mutation. The 4q21 genomic duplication in Patient B-1 included all of *SNCA* and *MMRN1*, whereas the duplicated region in Patient A-13 contained all of *SNCA* but only part of *MMRN1*. The *SNCA* triplication in the Iowa family also contains *MMRN1*, suggesting that overexpression of MMRN1 plays a role in cognitive deficit.

However, northern blotting analysis indicates a paucity of expression for *MMRN1* in neurons.[30] It therefore is unlikely that the effects of *MMNR1* are related to the development of dementia. *MMRN1* more likely plays a role in hemostasis and if vasogenic factors, including platelets and endothelial cells, are involved in dementia, *MMRN1* overexpression may still contribute to the dementia phenotype.

Previous studies reported the association of cardiac denervation and parkinsonism caused by *SNCA* gene triplication.[31] Low H/M ratios by [^{123}I]MIBG myocardial scintigraphy were reported in patients with sporadic PD.[32,33] In contrast, the H/M ratio was not decreased in patients with *parkin* mutations who lacked LBs in the autopsied brains.[34] In this regard, this finding is similar in patients with *SNCA* multiplication.

This study showed that the disease penetrance of Family A was 33.3%. The current ages of the asymptomatic carriers in this family are beyond the mean age at onset of patients. Thus, the difference may be caused by the *SNCA* expression levels between patients and asymptomatic carriers. Considering the multiple copies of *SNCA*, the expression level could be important. Indeed, double expression level of this protein compared with the normal brain was identified in Iowa family with *SNCA* triplication.[19] In addition, several haplotypes in the promoter region of SNCA including the sequence repeat element Rep1 were shown to associate with increased risk for sporadic PD.[35,36] However, whether the promoter alleles are risk factors for the development of PD is currently controversial.

Recently, Mueller and colleagues reported that single nucleotide polymorphisms located within the 3′side of exons 5 and 6, but not promoter polymorphism, correlated significantly with PD.[35] However, the functional association between PD and the associated region of SNCA remains unclear. In our study, the presence of asymptomatic carriers indicated that not only *SNCA* dosage but also another genetic variability in *SNCA* may be a risk factor for the development of PD.

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Pharmacokinetics of L-dopa

Special reference to food and aging

Abstract According to our data in rats, peripheral 3.4-dihydroxyphenylalanine (DOPA) kinetics are similar to striatal DOPA and dopamine kinetics. The measurement of plasma l-3.4-dihydroxyphenylalanine (L-dopa) concentration is thus useful to predict dopamine kinetics in the striatum and to treat the motor fluctuations of parkinsonian patients. In patients with Parkinson's disease (PD), long-term L-dopa therapy accelerated DOPA absorption and steepened features of L-dopa pharmacokinetics. In the senile-onset group, the pharmacokinetic pattern did not change even after long-term L-dopa therapy. The frequency of motor fluctuations is much lower in senile-onset patients with PD than in middle-onset patients. Differences in the pattern of L-dopa pharmacokinetics in the two groups may explain why the senile-onset group rarely develops 'wearing-off', even after long-term L-dopa therapy. L-dopa is transported by a saturable active transporter system, called the LNAA (large neutral amino acid) system, in the gut and blood brain barrier. L-dopa absorption is thus affected by food intake, especially a protein-rich diet. The slope of the time-concentration curve for L-dopa administered before a meal is steeper than if it is administered after a meal. Considering that pulsative stimulation of L-dopa may cause motor fluctuations, L-dopa should be given after meals whenever possible, even if it necessitates a higher L-dopa dose.

Key words Parkinson's disease · absorption · LNAA system · L-dopa · aging

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Introduction

L-dopa is the gold standard of antiparkinsonian pharmacotherapy; however, motor fluctuations, such as 'wearing-off', often develop during long-term L-dopa therapy. The definition of wearing-off is fluctuation of parkinsonian symptoms in line with L-dopa pharmacokinetics [1]. Therefore, the pharmacokinetics of L-dopa are very important in treating patients with Parkinson's disease (PD). The half-life ($T_{1/2}$) of L-dopa is short (1 h) and its absorption is greatly influenced by food intake and aging. Thus, these factors make PD therapy complicated.

Because L-dopa is used with a DOPA decarboxylase inhibitor (DCI), catechol-O- methyltransferase (COMT) is an important enzyme influencing peripheral L-dopa metabolism and L-dopa effects on parkinsonian symptoms. Therefore, nowadays, knowledge about L-dopa pharmacokinetics is increasingly important in treating PD.

In the present review, DOPA and dopamine kinetics in blood and brain, and food and aging effects on the pharmacokinetics of L-dopa are discussed. The correlation between DOPA and 3-O-methyl DOPA is also featured.

L-dopa and dopamine kinetics in peripheral blood and striatum

L-dopa pharmacokinetics are very important when treating PD, and the L-dopa concentration can be measured in blood. Dopamine kinetics in the brain are more

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critical than peripheral DOPA kinetics; however, it is very difficult to measure dopamine in the brain of PD patients in vivo. Therefore, it is important to know how closely peripheral L-dopa kinetics reflect dopamine kinetics in the brain, especially in the striatum.

We measured DOPA and dopamine concentrations in the blood and striatum of normal rats after single and repeated L-dopa administration (Fig. 1) [2]. DOPA and dopamine kinetics in the striatum were well correlated with peripheral DOPA kinetics. We also showed that repeated L-dopa (L-dopa 50 mg/kg + benserazide 12.5 mg/kg) administration for 28 days increased the area under the concentration-time curve (AUC) and shortened $T_{1/2}$ and time to maximum plasma concentration (T_{max}) for both peripheral DOPA and central DOPA and dopamine in normal rats.

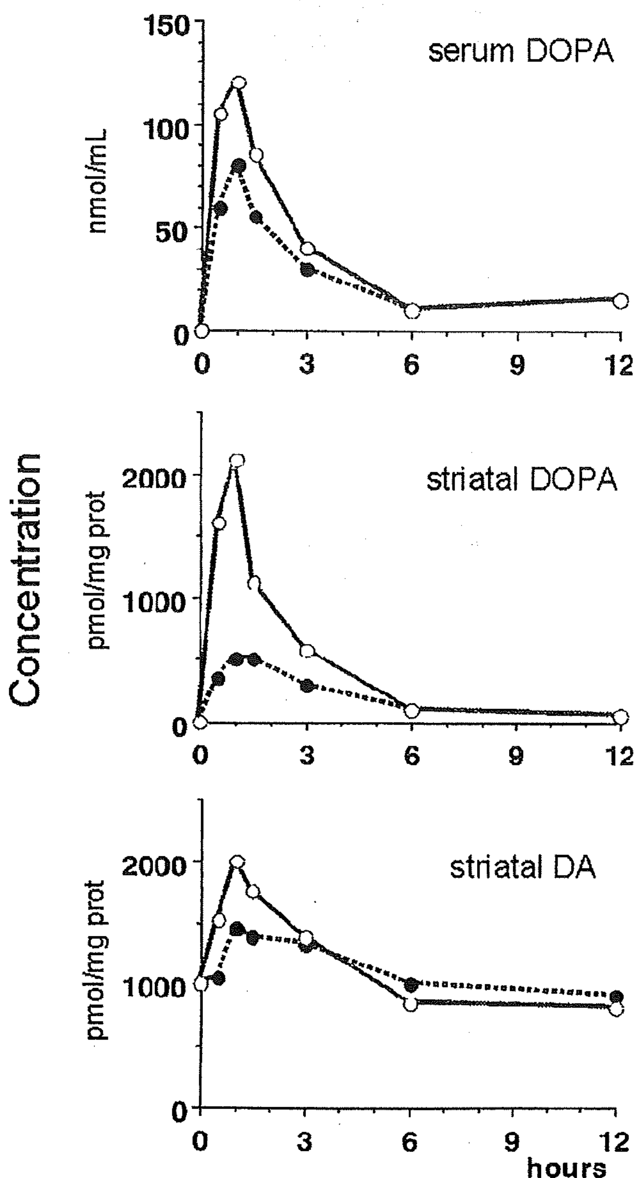


Fig. 1 Time course of serum L-dopa and striatal DOPA and dopamine (DA) after single and repeated administration of L-dopa in rats (-----●----- single administration, ——○—— repeated administration). Time course of serum L-dopa concentration is similar to that of striatal DOPA and dopamine. Repeated L-dopa administration increases C_{max} and AUC and shortens $T_{1/2}$

L-dopa pharmacokinetics in PD patients

PD patients were given L-dopa 100 mg plus benserazide 25 mg orally at 8:00 am after an overnight fast. Plasma DOPA concentrations were measured prior to treatment (baseline) and at 15 min, 30 min, 1 h, 2 h, 3 h and 4 h after medication using HPLC-ECD (L-DOPA test) [3]. In PD patients (onset age < 60 years old) who had received L-dopa therapy for longer than 5 years, the $T_{1/2}$ and T_{max} of L-dopa were much shorter than those measured in PD patients with a duration of L-dopa therapy of less than 5 years (Fig. 2a). In addition, the AUC was greater in the longer therapy duration group (Fig. 2a). These changes in the pharmacokinetics of L-dopa were significantly correlated with duration of L-dopa therapy and dose of L-dopa. A 4-year longitudinal study showed that four of five patients displayed an increased AUC and shortened $T_{1/2}$ and T_{max} at the second assessment [4]. Patients who demonstrated the wearing-off phenomenon had a significantly higher maximum plasma concentration (C_{max}) and greater AUC, and significantly shorter $T_{1/2}$ and T_{max} than those who did not display wearing-off. The pattern of L-dopa kinetics in those with wearing-off was obviously steeper than that of patients without wearing-off.

It is reasonable to suppose that these changes in pharmacokinetic features are due to changes in absorption or metabolism of L-dopa. Decreased metabolism of L-dopa can explain the increase in AUC and C_{max}, but cannot explain the shortening of T_{max} and $T_{1/2}$. Increased absorption, however, can explain the increase in AUC and C_{max} and the shortening of T_{max}. If the absorption system is saturable, increased absorption can also explain the shortening of $T_{1/2}$. L-dopa is transported by the saturable active transport system called the LNAA (large neutral amino acid) system in the gut and blood brain barrier (BBB) [5]. Furthermore, intravenous administration has demonstrated that the distribution and elimination of L-dopa was not changed after long-term L-dopa therapy [6]. Both monoamine oxidase (MAO) activity and COMT activity in the brain are unaffected by long-term L-dopa administration [2, 7]. Therefore, our results show that long-term L-dopa therapy alters its own kinetics by increasing the absorption of L-dopa. As early as 1971, Abrams et al. reported that long-term L-dopa therapy increases its own absorption [8]. At that time, L-dopa therapy involved L-dopa administered without a DCI, and liver DOPA decarboxylase (DDC) ac-

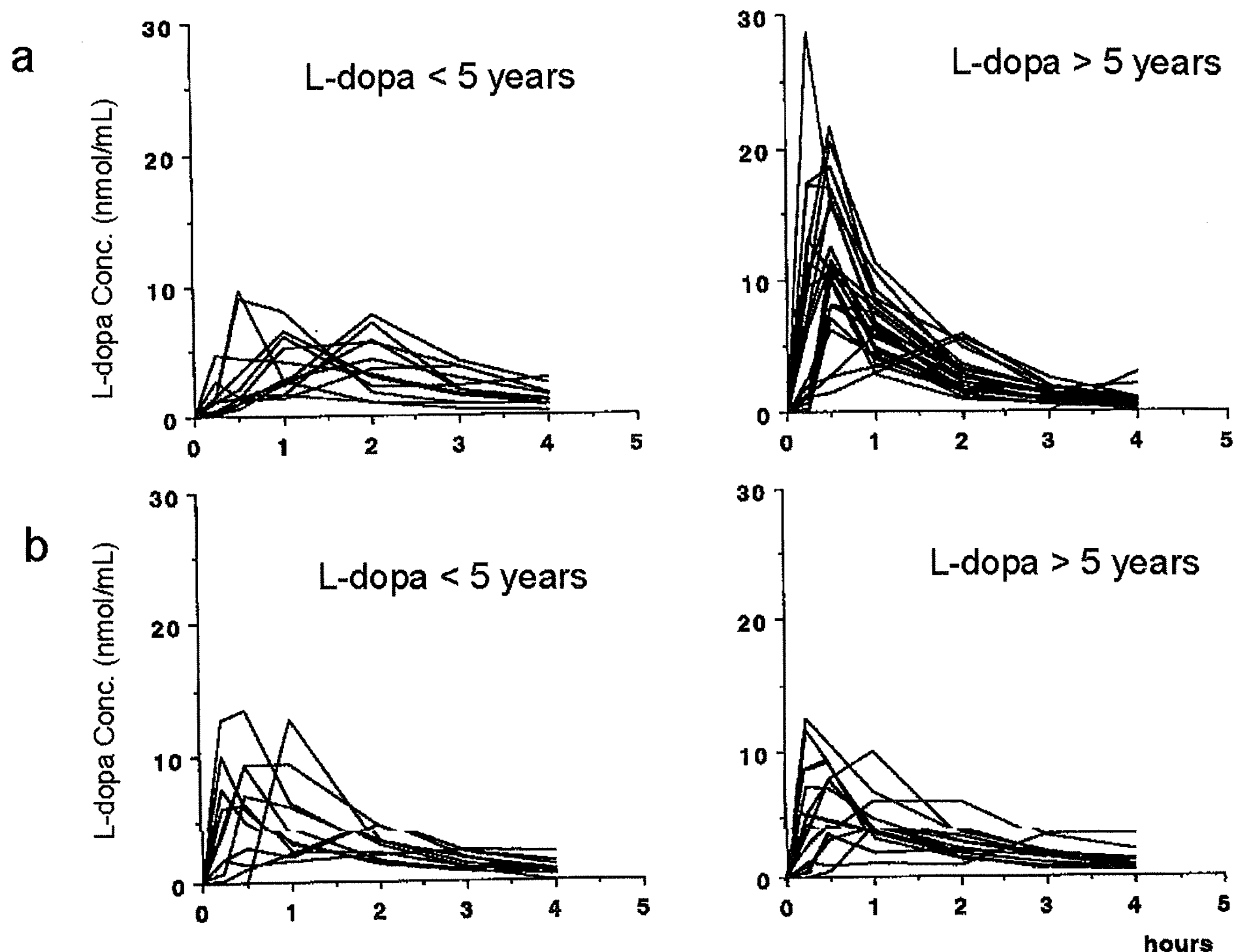


Fig. 2 L-dopa pharmacokinetics in parkinsonian patients with disease onset at < 60 years of age (**a**) and > 60 years of age (**b**) according to duration of L-dopa therapy. Long-term L-dopa therapy (> 5 years' duration) increases C_{max} and AUC and shortens $T^1/_2$. Pharmacokinetic changes after long-term L-dopa therapy are not seen in the senile-onset group

tivation was suggested as a cause of this phenomenon [9]. In fact, long-term L-dopa administration activates DDC in the liver but not in the brain, and no data has been published in the gut [10]. Our data was obtained using L-dopa with a DCI. It has been reported that plasma DDC is induced by administration of L-dopa with a DCI [11]. Therefore, the DDC activation theory cannot explain our results. We propose that long-term L-dopa therapy may induce the LNAA transporter system.

Aging and L-dopa pharmacokinetics

Although long-term L-dopa therapy steepened L-dopa kinetics, this change was less marked in senile-onset patients (onset age > 60 years old) than in younger onset patients (Fig. 2b). The frequency of wearing-off is much lower in senile-onset patients than in younger onset patients [12]. This suggests that changes in peripheral L-dopa pharmacokinetics after long-term therapy certainly contribute to the clinical expression of wearing-off.

Food and acidity effects on L-dopa absorption

L-dopa shares a saturable transporter system with other LNAA such as phenylalanine. Therefore, competitive inhibition of L-dopa absorption occurs with rising concentrations of neutral amino acids derived from food (Fig. 3). The L-dopa pharmacokinetic profile is steeper when intake occurs before a meal than after a meal. Considering that pulsative stimulation of L-dopa may cause motor fluctuations, L-dopa should be given after meals

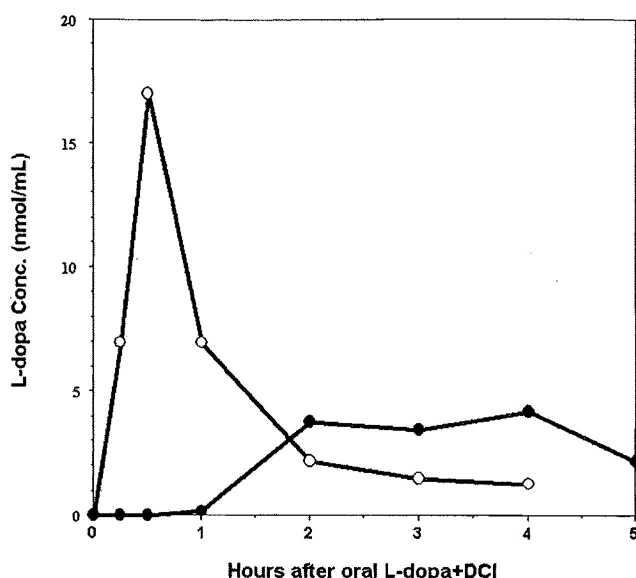


Fig. 3 Effects of a meal on L-dopa kinetics in a 55-year-old female patient with Parkinson's disease (○ L-dopa administration before meal, ● L-dopa administration after meal). C_{max} and AUC were markedly decreased and T_{max} was increased by L-dopa administration after a meal

whenever possible, even if it necessitates a higher L-dopa dose.

L-dopa is known to be easily soluble in acid environments and the pH of gastric juices affects the absorption of L-dopa. Fig. 4 shows the results of an L-DOPA test from a 65-year-old male patient with PD. The first test was performed using the ordinary method and, 1 year later, the second test was performed using duodenal infusion of L-dopa. Although the pH of duodenal juice is high, absorption was not impaired because L-dopa was administered dissolved in water. When it is dissolved in water, L-dopa is absorbed rapidly and adequately, even in alkaline duodenal juice.

L-dopa and 3-O-methyl DOPA

The main metabolite of DOPA is dopamine, formed by decarboxylation, and the COMT pathway is usually a rather minor pathway in the metabolism of DOPA. However, when L-dopa is used with a DCI, the COMT pathway is activated and a large amount of 3-O-methyl DOPA (3OMD) is synthesized. The $T_{1/2}$ of 3OMD (16 h) is much longer than that of DOPA; thus, the plasma concentration of 3OMD increases according to long-term L-dopa therapy (Fig. 5). Although the plasma concentration of 3OMD is usually closely correlated to daily L-dopa dose (Fig. 6), some patients show very low 3OMD concentrations relative to the L-dopa dose and plasma L-dopa concentration. These patients may obtain a good response with a COMT inhibitor. As COMT inhibitors will be approved for PD therapy in Japan this year, it will be important to assess this hypothesis soon.

3OMD also uses the LNAA transporter system in the gut and BBB. After protein-rich meals, competition between L-dopa, dietary LNAA and 3OMD for gut and BBB transport may further contribute to motor fluctuations.

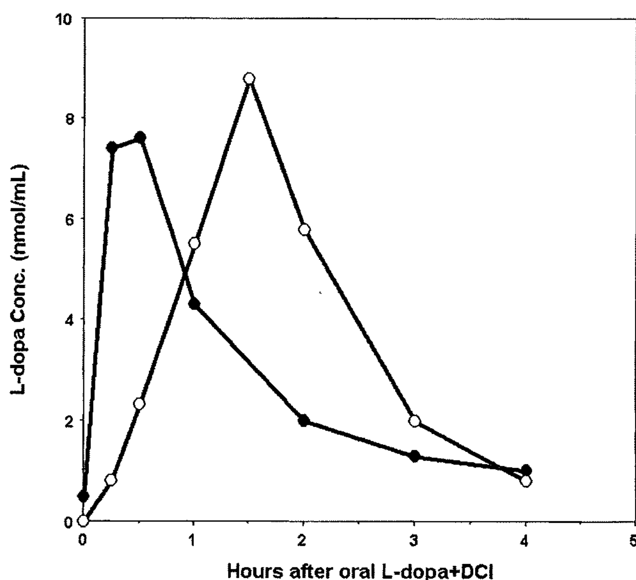


Fig. 4 Effects of duodenal infusion of L-dopa in a 65-year-old male patient with Parkinson's disease (○ L-dopa administration by tablet orally, ● L-dopa administration by duodenal infusion in water suspension). L-dopa concentration is rapidly and adequately increased by duodenal infusion

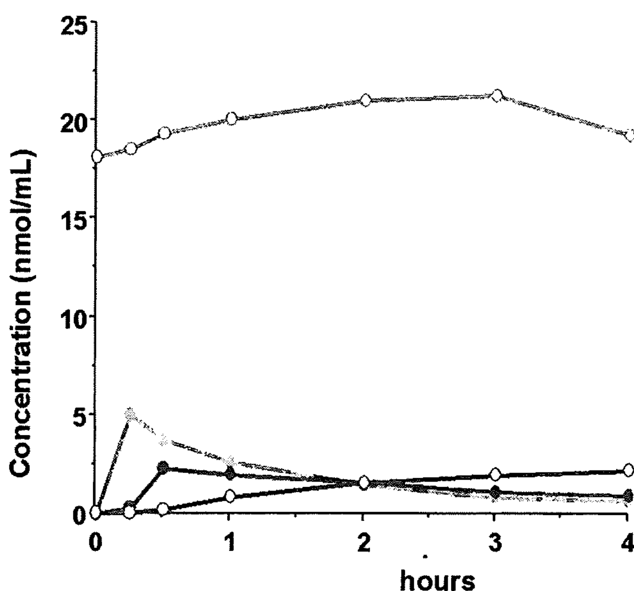


Fig. 5 Plasma concentration of dopa and 3OMD in PD patients (○ concentration of 3OMD, ● concentration of dopa, red line: long-term L-dopa therapy, blue line: L-dopa initial use)

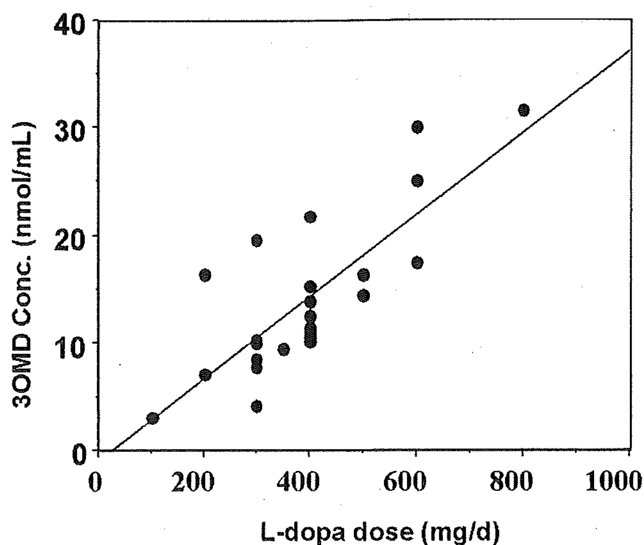
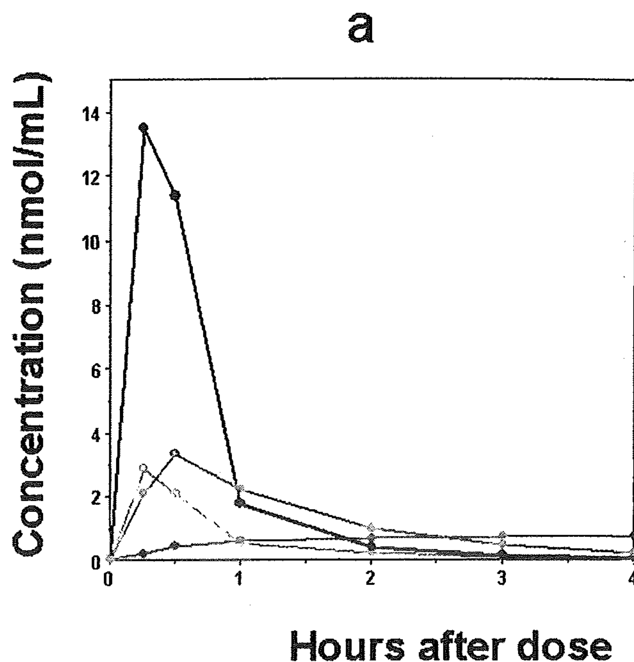


Fig. 6 Relationship between L-dopa daily dose and plasma concentration of 3OMD in PD patinets

Following L-dopa administration without a DCI, the plasma dopamine concentration is greatly increased and the 3OMD concentration is very low; however, L-dopa administration with a DCI results in synthesis of a large amount of 3OMD in plasma (Fig. 7) by activating the COMT pathway. If L-dopa is administered in combination with both a DCI and a COMT inhibitor, new metabolic pathways may be activated such as enhanced quinine formation [13].

Conclusion

Peripheral L-dopa kinetics closely reflects dopamine kinetics in the striatum so that measurements of L-dopa kinetics are useful for treating patients with PD. L-dopa is transported by a saturable active transporter system (LNAA system) in the gut and BBB. Onset age of PD, treatment duration, and food are greatly influence peripheral L-dopa kinetics. Food and onset age decrease C_{max} and prolong T_{max} and $T_{1/2}$. When L-dopa is administered with a DCI, the COMT pathway is activated and the COMT inhibitor becomes an important factor for peripheral L-dopa kinetics.



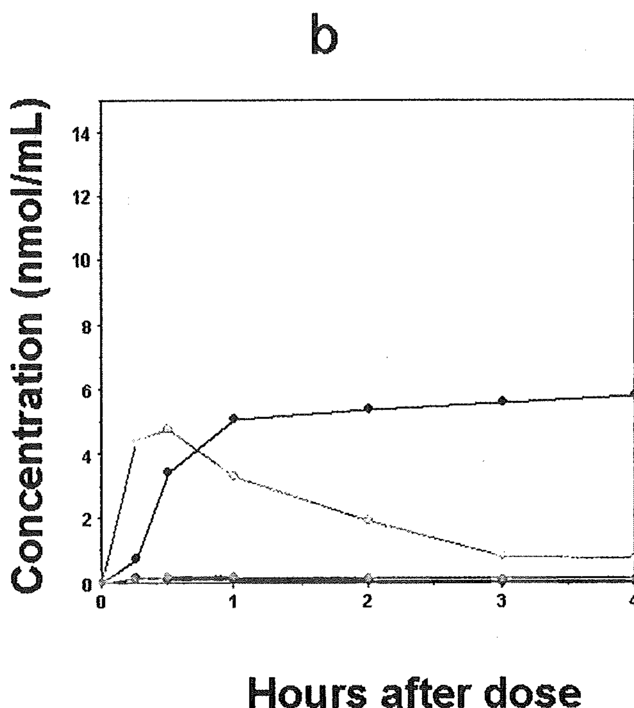


Fig. 7 Plasma concentration of DOPA and its metabolites after oral administration of L-dopa (100 mg) without DCI (**a**), and oral administration of L-dopa (100 mg) with DCI (benserazide 25 mg) (**b**) of the same PD patient (red: DOPA; black: dopamine; blue: 3OMD; green: homovanillic acid (HVA))

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