To minimize the influence of neuronal loss on semiquantitative analysis, we selected specimens containing at least 100 remaining neurons as described previously [33]. All sections obtained from the enrolled subjects and used for this analysis fulfilled this criterion. Two independent observers blinded to the clinical information counted the numbers of hOGG1-2a-positive neurons in SN and PN. Differences between groups were examined for statistical significance using one-way ANOVA. A P value less than 0.05 was considered significant.

Subcellular fractionation

Fresh frozen brain including midbrain (five PD and five control subjects) and pons (two PSP, two CBD and three control subjects) were obtained (Table 1). Approximately 0.5 g of frozen brain blocks were placed into 3.5 ml ice-cold homogenization buffer [0.32 M sucrose and 4 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES)-NaOH, pH 7.4] and homogenized using a Potter-Elvehjem homogenizer (9 up-and-down strokes, 900 rpm) in the presence of a mixture of protease inhibitors (Complete Mini EDTA-free, Roche Diagnostics, Penzberg, Germany). Nuclear, mitochondrial, microsomal, and cytosolic fractions were obtained using the methods described previously [15, 29].

Western blot analysis of hOGG1-2a

Each sample was separated by 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis. The separated proteins were transferred onto a polyvinylidene difluoride (PVDF) microporous membrane (Bio-Rad, Hercules, CA) using transfer buffer (40 mM CAPS, 30 mM TRIS, and 15% methanol). The transferred membrane was blocked with 5% skim milk and incubated overnight with primary antibodies at 4°C. After incubation with HRP-conjugated secondary antibodies, the reaction was visualized using a chemiluminescence reagent. The intensity was analyzed by LAS-1000plus (Fuji film, Tokyo, Japan).

Results

Regional expression of hOGG1-2a

Control subjects

In the control subjects, hOGG1-2a immunoreactivity was rarely observed in any brain region examined including cortex, basal ganglia, SN (Fig. 1A), and PN (Fig. 1E), and never observed in glial cells. However, the number of hOGG1-2a-positive neurons in SN increased with age (Fig. 2A). Western blot analysis also showed that expression of hOGG1-2a increased with age in control subjects (Fig. 3A, C). These results are consistent with those reported previously [5, 7].

Parkinson's disease

All PD patients showed moderate to severe neuronal loss in the SN. While the remaining nigral neurons of short-duration PD group showed intense cytoplasmic immunostaining with granular pattern for hOGG1-2a (Fig. 1B), those of the long-duration group did not show intense cytoplasmic immunostaining. Double-immunofluorescence staining for hOGG1-2a and TH showed immunoreactivity for hOGG1-2a in TH-positive neurons of the SN (Fig. 1I–K), suggesting increased hOGG1-2a in dopaminergic neurons of the SN. Lewy bodies did not stain with hOGG1-2a. There was no immunoreactivity in the nuclei. Interestingly, the immunoreactivity was barely seen in the cortex, basal ganglia, and PN (Fig. 1F). There was no immunoreactivity in glial cells. Semiquantitative analysis showed that the percentage of hOGG1-2a-positive neurons in the SN was higher in PD than in aged-matched control, PSP, and CBD cases. Repeated paired analyses with Bonferroni's correction showed significant differences between PD and aged-matched control ($P < 0.05$), but no significant differences between PSP, CBD and aged-matched control ($P > 0.05$) (Fig. 2B). Semiquantitative analysis also showed that the expression of hOGG1-2a was significantly higher in the short-duration PD group relative to the aged-matched control. In the long-duration PD group, the number of hOGG1-2a-positive neurons was slightly higher, albeit statistically insignificant from the aged-matched control (Fig. 2C). Western blot analysis demonstrated up-regulation of hOGG1-2a in the SN of PD compared with age-matched controls. The level of hOGG1-2a in SN of PD was 1.6- to 2.9-fold higher than age-matched controls (cases 9, 10, 12) (Fig. 3B).

Progressive supranuclear palsy and corticobasal degeneration

In PSP and CBD patients, severe neuronal loss was noted in basal ganglia and SN but not in PN. Immunoreactivity for hOGG1-2a was observed in limited regions such as PN (Fig. 1G, H). Immunostaining showed cytoplasmic granular pattern without nuclear staining in pontine neurons, but no obvious immunoreactivity in the cortex, basal ganglia, and SN (Fig. 1C, D). Glial cells including oligodendrocytes and astrocytes were barely immunoreactive in PSP and CBD. Semiquantitative analysis showed that the percentage of hOGG1-2a-positive neurons in PN was higher in PSP and CBD than aged-matched control and PD. Repeated paired analyses with Bonferroni's correction showed significant differences between PSP, CBD and aged-matched control ($P < 0.05$) (Fig. 2D). Western blot analysis showed 3.9- to 4.9- and 2.8- to

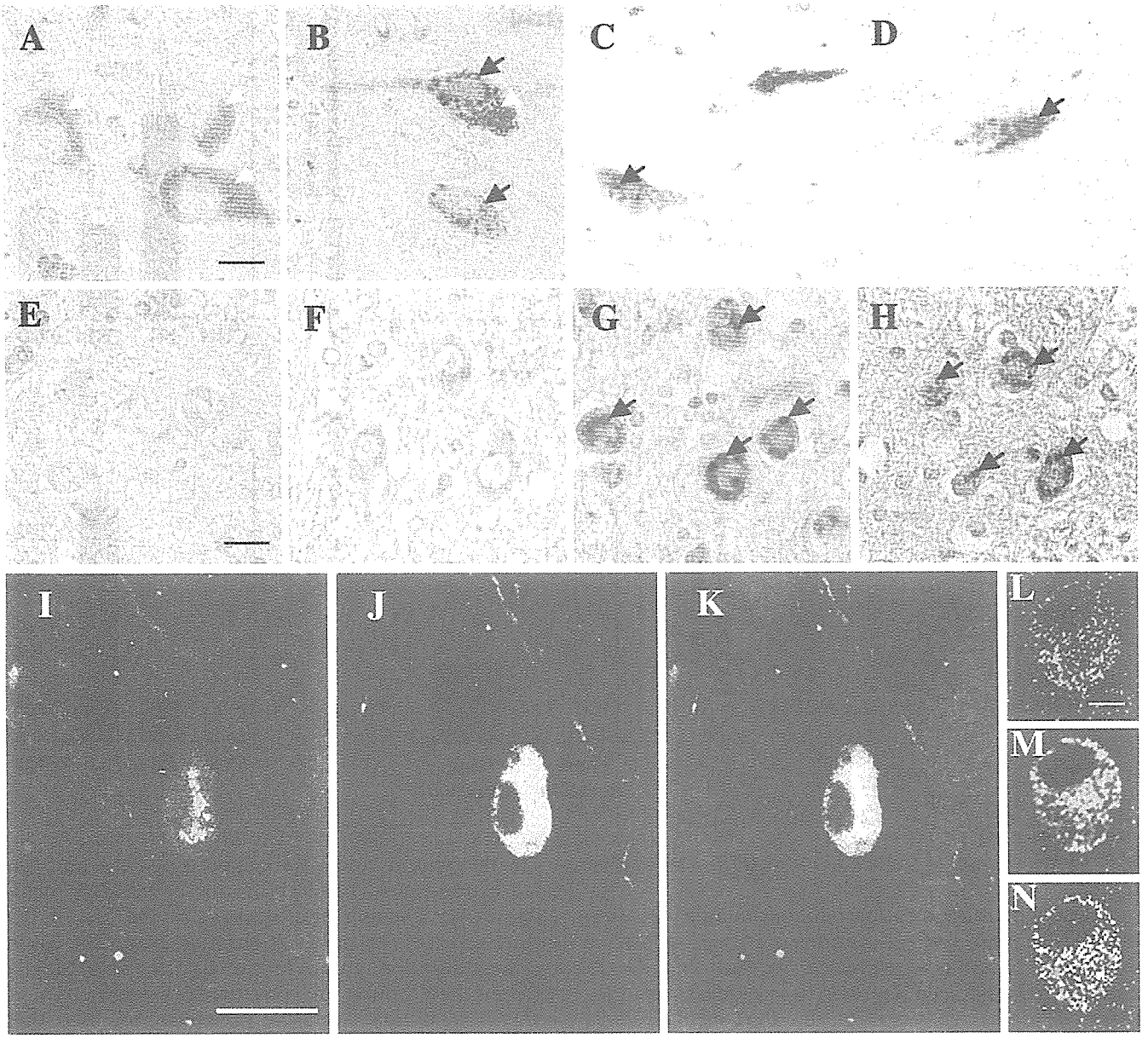

Fig. 1 Immunohistochemistry for hOGG1-2a in the SN (**A**–**D**) and PN (**E**–**H**) in representative subjects. **A**, **E** Control; **B**, **F** PD; **C**, **G** PSP; **D**, **H** CBD. Note the granular staining for hOGG1-2a (*arrow*) in the cytoplasm of SN neurons in the PD patient. Neuromelanin (*white arrow*). Note also the granular staining for hOGG1-2a in the cytoplasm of pontine neurons in PSP and CBD (*arrows*). **I**–**N** Neurons in SN of PD patient double stained with anti-hOGG1-2 antibody (*red* in **I**), anti-TH antibody (*green* in **J**) and merge (**K**), or with anti-hOGG1-2 antibody (*red* in **L**), anti-cytochrome oxidase subunit I antibody (*green* in **M**) and merge (**N**). Note the granular pattern of hOGG1-2a expression in the cytoplasm in TH-positive neurons and colocalization with cytochrome oxidase subunit 1 (*hOGG* human 8-oxoguanine DNA glycosylase, *SN* substantia nigra, *PN* pontine nuclei, *PD* Parkinson's disease, *PSP* progressive supranuclear palsy, *CBD* corticobasal degeneration, *TH* tyrosine hydroxylase). *Bars* **A**–**H** 10 μm; **I**–**K** 20 μm; **L**–**N** 10 μm

5.8-fold higher expression of hOGG1-2a in the pons of PSP and CBD than age-matched controls (casea 9, 10) (Fig. 3C, D), respectively.

Subcellular localization of hOGG1-2a

Since immunohistochemical studies could not distinguish between the precursor and processed forms of hOGG1-2a (the former but not the latter possesses a mitochondrial targeting signal consisting of 23 amino acid residues [23]), we performed subcellular fractionation study using SN from PD patients. hOGG1-2a is initially translated as a 43-kDa precursor molecule with the mitochondria targeting signal at the N-terminal end. After translocation into the mitochondria, the 43-kDa precursor is processed to a 40-kDa mature hOGG1-2a. As expected, we detected 40- and 43-kDa bands in mitochondrial and cytosolic fractions, respectively. However, the signal in the mitochondrial fraction was 1.4-fold stronger than in cytosolic fraction (Fig. 3E). We used TOM40 as the mitochondria marker, which is located in the outer membrane of the mitochondria [14,

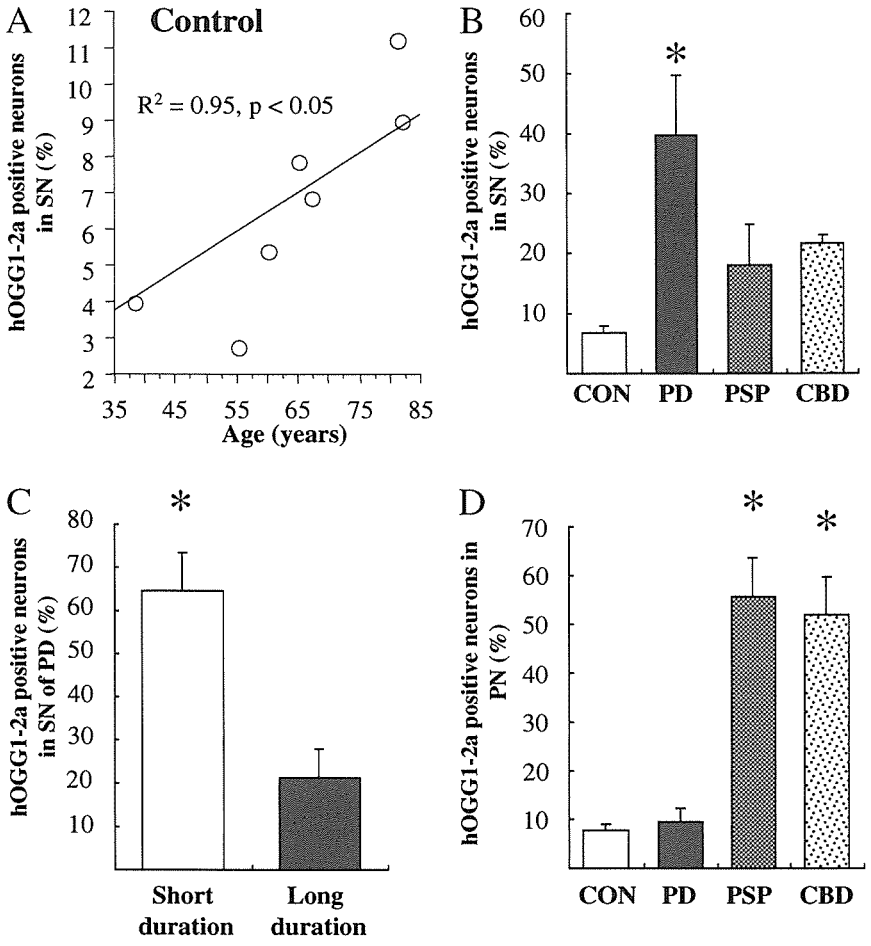

Fig. 2 Results of semiquantitative analysis. **A** Age-dependent increase in the percentage of hOGG1-2a-positive neurons in SN of control subjects. There was a significant correlation with age ($P < 0.05$). **B, D** Results of semiquantitative analysis of hOGG1-2a in SN and PN. The percentages of hOGG1-2a-positive neurons (mean ± SEM) were significantly higher in SN of PD and PN of PSP and CBD than the control (*CON*) (*$P < 0.05$, one-way ANOVA and Bonferroni's correction). **C** Percentage of hOGG1-2a-positive neurons in short- and long-duration PD groups (mean ± SEM). The percentage of hOGG1-2a-positive neurons was significantly higher in the short-duration group than in long-duration group (*$P < 0.05$)

25]. There was no signal for hOGG1-2a in the nuclear fraction.

We also performed double immunostaining with anti-hOGG1-2a and anti-cytochrome oxidase subunit I antibodies. Immunofluorescence for anti-hOGG1-2a antibody also showed a granular pattern in the cytoplasm of nigral neurons (Fig. 1L). Part of hOGG1-2a molecules were colocalized with cytochrome oxidase I, suggesting that hOGG1-2a that colocalized with cytochrome oxidase I in the mitochondria is the 40-kDa processed molecule from the 43-kDa precursor (Fig. 1L–N).

Discussion

Mitochondria are intracellular organelles in which ATP is synthesized, and such synthesis requires oxygen. mtDNA is more vulnerable to oxidative stress than nuclear DNA [18, 26], because mtDNA is located in the inner mitochondria membrane in which electron transport chain generates ROS. Since mtDNA has no intron, mtDNA mutations could lead to amino acid replacement that could induce mitochondrial dysfunction(s). Therefore, mtDNA repair enzymes are important to maintain mitochondrial functions.

In the present study, we demonstrated the up-regulated expression of hOGG1-2a, one of mtDNA repair enzymes, in dopaminergic neurons in the SN of PD brains, especially in the short-duration group but not in the long-duration group of the disease, indicating a different time course of compensatory mechanism of mtDNA oxidation. We have previously reported that MutT homolog (MTH1), an enzyme known to play an important role in controlling spontaneous mutagenesis in mtDNA, was up-regulated in the SN neurons of PD brains but not in other related neurodegenerative disorders such as multiple system atrophy [29]. Thus, overexpression of such repair enzymes could be a common event in the process of PD. What does hOGG1-2a up-regulation mean? The most plausible explanation is that hOGG1-2a is up-regulated secondarily to mtDNA oxidative damage to protect neurons form mutagenesis. Indeed, overexpression of hOGG1 within the mitochondria enhances the repair of mtDNA errors and rescues the cells from oxidative stress [9, 24].

Apart from PD, we detected intense hOGG1-2a immunoreactivity only in PN, while we could not detect hOGG1-2a immunoreactivity in the regions of severe neuronal loss, such as SN and frontal cortex, in PSP and CBD brains. Why were there regional differences in the expression of hOGG1-2a in such diseases? It is possible

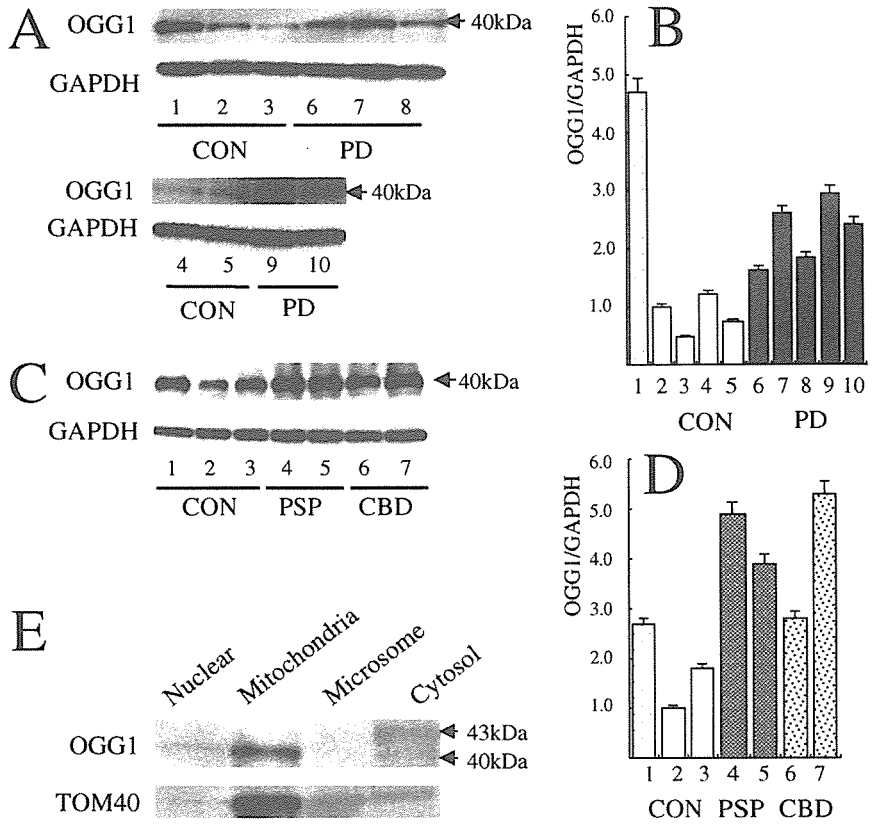

Fig. 3 **A** Immunoblotting of hOGG1-2a in midbrain sections of an elderly control subject (*lane 1*), younger control subject (*lane 4*), three control subjects age-matched for patients with PD (*lanes 2, 3, 5*), and five PD patients (*lanes 6–10*). **B** Quantitative analysis of hOGG1-2a expression levels normalized to GAPDH in control (*CON*) and PD. **C** Immunoblotting of hOGG1-2a in the pons of an elderly control subject (*lane 1*), two control subjects aged-matched for PSP and CBD patients (*lanes 2* and *3*), two PSP patients (*lanes 4* and *5*) and two CBD patients (*lanes 6* and *7*). **D** Quantitative analysis of hOGG1-2a expression levels normalized to GAPDH in control, PSP and CBD. **E** Analysis of subcellular fractions in the midbrain of PD patient

that the topographic differences reflect the course of the compensatory mechanism for mtDNA oxidation as observed in the different stages of PD. PSP and CBD are progressive neurodegenerative disorders characterized by extensive neuronal degeneration in multiple subcortical regions such as basal ganglia and brainstem nuclei [8, 16]. Among the vulnerable regions, PN is one of the affected lesions. In PSP, the appearance of neurofibrillary tangles (NFTs) and occasional neuronal loss are noted in PN [8, 17]. On the other hand, there is almost no neuronal loss and NFTs in PN of CBD [8, 10]. The characteristics of CBD in PN are tau inclusions in glia and cell processes [8]. However, abnormal tau and NFTs were found in pontine neurons in CBD [22], suggesting such neurons are also affected. Although the pathological findings in PN are different between PSP and CBD, our results showed that the expression pattern of hOGG1-2a in PSP was similar to that in CBD, suggesting both disorders have some common neurodegenerative mechanism. The immunoreactivities for hOGG1-2a in the PN of PSP and CBD indicated that oxidative stress occurred in the PN neurons. Ample evidence indicates that mitochondrial impairment and oxidative stress are associated with NFTs formation and neurodegeneration [2]. Thus, mitochondrial dysfunctions and oxidative stress might be related to the pathogenesis of PSP and CBD [2, 3, 6, 30]. Indeed, complex I activity was reduced in a cybrid line that originated from PSP patients [30]. Although there is no report of mitochondrial dysfunction in CBD so far, considering the common biological background including tauopathies, it is possible that mitochondrial dysfunction is involved in the pathogenesis of CBD.

In the control subjects, the proportion of hOGG1-2a-positive neurons increased with age. In addition, Western blot analysis revealed high expression of OGG1-2a in the normal control at the age of 89 years. In human brain of patients older than 70 years, the amount of 8-hydroxy-2'-deoxyguanosine (8-OHdG) in mtDNA is 15-fold greater than in patients < 70 years of age [18]. In other human organs (heart and diaphragm), 8-OHdG levels in mtDNA also increased exponentially with age [12, 13]. These results suggest that oxidative stress is increased significantly in old age rather than young age. The significant increase in hOGG1-2a in old age is consistent with response to oxidative stress associated with aging.

The up-regulation of mtDNA repair enzymes might overcome the oxidative insults in the brain. Furthermore, it is conceivable that up-regulation of mtDNA repair enzymes could be a potentially beneficial target for gene therapy aiming at neuroprotection in PD [9, 24]. Although the primary cause of neurodegeneration remains to be elucidated, our results indicate the importance of oxidative stress and mitochondrial dysfunction in the pathogenesis of neurodegenerative disorders.

In conclusion, we showed here that hOGG1-2a is up-regulated in limited areas of the brain lesion, i.e., SN of PD patients and PN of PSP and CBD patients. This selectivity suggests that up-regulation of hOGG1-2a is a

secondary response to neurodegenerative process, probably due to oxidative stress in the mitochondria.

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Clinicogenetic study of *PINK1* mutations in autosomal recessive early-onset parkinsonism

Abstract—The authors performed *PINK1* mutation analysis of 51 families with autosomal recessive Parkinson disease (ARPD). They found two novel *PINK1* mutations: one was a homozygous deletion (13516-18118del) and the other a homozygous missense mutation (C388R). Clinically, the patients with the deletion had dementia. Thus, early-onset PD with dementia may be considered *PINK1*-linked parkinsonism. Furthermore, patients with *PINK1* mutations form 8.9% of *parkin*- and *DJ-1*-negative ARPD families.

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To date, six genes have been identified as the causative genes for familial forms of Parkinson disease (PD). All the causative genes are proven causes of PD except for *UCH-L1*. The *alpha-synuclein*[1] and *PARK8*[2] are the causative genes for autosomal dominant PD, and the *parkin*,[3] *DJ-1*,[4] and *PINK1*[5] are the causative genes for autosomal recessive PD (ARPD). Among the monogenic forms of PD, mutations of *parkin* have been detected in approximately 50% of families with ARPD.[6] In contrast, *DJ-1* mutations are rare in ARPD.[7] Recently, *PINK1* was detected as the causative gene for PARK6 in Italian and Spanish families.[5] We recently reported six novel point mutations in *PINK1* in Japanese, Israeli, Philippine, and Taiwanese families.[8] Thus, this mutation appears to be distributed worldwide. In the present study, we performed extensive mutation analyses for *PINK1* in 51 families with ARPD negative for *parkin* and *DJ-1* mutations.

Methods. Blood samples and clinical information were obtained from the neurologists. Diagnosis of PD was made by the participating neurologists. We investigated 51 ARPD families (56 patients; male 28, female 28, aged 9 to 80 years, mean 47 years) from nine countries including 26 Japanese, 11 Canadian, 5 Taiwanese, 4 Israeli, 1 Tunisian, 2 Korean, 1 Turkish, and 1 Bulgarian. In the present study, the subjects were either from families of consanguineous marriages or at least two affected siblings in the same generation, and we also included a single patient with early-onset parkinsonism with homozygosity in *PARK6* region in haplotype analysis. The study was approved by the Ethics Review Committee of Juntendo University. Blood samples for genetic analysis were collected after obtaining informed consent from 56 patients from 51 families. DNA was prepared using standard methods. None of the subjects had mutations in *parkin* and *DJ-1*.

We investigated 56 patients from 51 families for *PINK1* mutations.[3,8] For sequence analysis, the coding exons of *PINK1* were amplified by PCR using published primers.[8] We also performed direct sequencing of all coding exons of *DJ-1*. Dideoxy cycle sequencing was performed with Big Dye Terminater Chemistry (Applied Biosystems, Foster City, CA). This was followed by exon sequencing on ABI377 and 310 automated DNA sequence analyzers (Applied Biosystems). We used the following primer to detect the breakpoint of deletion involving exons 6 to 8: forward 5'-AGACAGAATCTTGCTTTGTTGC-3', reverse 5'-TGGTTCTCC-CTAACGTCTCCT-3'.

Results. We found two novel *PINK1* mutations. The first mutation was a homozygous exonic deletion involving exons 6 to 8. In this family (Family A), no consanguinity was reported. We performed the mutation analysis based on the homozygosity of the haplotype analysis in this gene region. Subsequently, we identified the breakpoint of the deletional mutation (figure 1). The second mutation was a novel point mutation. A homozygous missense mutation (C388R) in exon 6 was detected in all the affected members of the family (Family B) (figure 2). The mutation is highly conserved across species. We did not find the same mutations in 300 chromosomes from normal Japanese population.

Clinically, all three patients were of a young age at onset (table) and had parkinsonism that showed good response to levodopa. All had hyperreflexia but no remarkable autonomic disturbances. Patient A1 with the deletional mutation showed long disease duration, sleep benefit, and dystonia at onset, similar to patients with *parkin* mutations.[6] Patient A1 also had various psychological disorders including dementia, depression, and hallucinations. Sleep benefit was observed in Patient B2, but not in Patient B1. Patients B1 and B2 with the missense mutation showed lack of dystonia at onset and psychological disorders.

Discussion. To our knowledge, all the *PINK1* mutations reported so far have been point mutations. In the case of *parkin*, exonic deletion mutations are more frequent than point mutations among the Japanese.[7] This relatively high mutation rate of the de-

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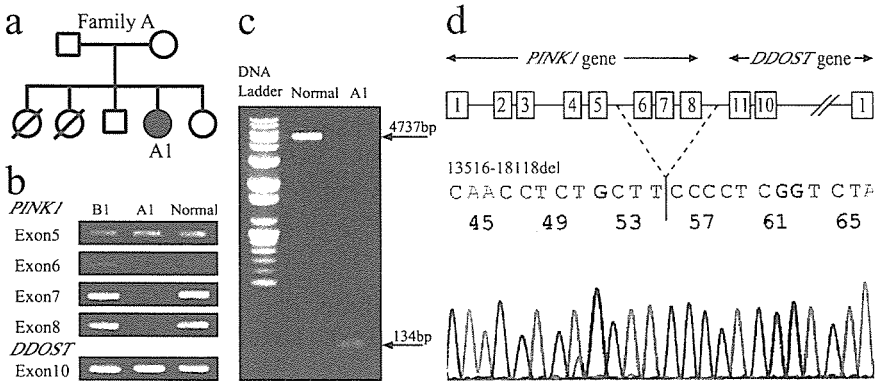

Figure 1. (a) Pedigree structure of Family A. (b) Patient A1 had exons 6 to 8 deletion. The DDOST *gene, which is located next to the* PINK1 *gene, showed no deletional mutation. Patient B1 and a normal control had no deletion. (c) Exons 6 to 8 PCR products: 4,737 bp, normal; 134 bp, exons 6 to 8 deleted. (d) Analysis of the sequences of the breakpoint regions revealed the exon 6 to 8 deletion. The sequenced deletion junctions did not reveal extensive homology such as repetitive elements to the deletion end point.*

letions may be related to the giant size of the 1.4-Mb *parkin* gene.[7] Considering the structure of *PINK1* spanning 18 kb,[5] *PINK1* deletional mutations may be more infrequent than *parkin* deletional mutations. The frequency of deletion formation correlates with the extent of homology between the short repeated sequences, although other factors may be involved. In our case, the deletion junctions sequenced did not reveal extensive homology such as repetitive elements to the deletion end point. A recent report identified a higher number of patients carrying a single heterozygous mutation in mostly sporadic early-onset parkinsonism (5%) than controls (1%).[9] Thus, the heterozygous deletional mutation may be a risk factor or it is possible that some patients with sporadic early-onset parkinsonism may have a single heterozygous deletion including this deletion. In this regard, it is important to look for the breakpoint not only to elucidate the mechanism of deletion but also to screen the deletions using PCR methods. Based on the semiquantitative analysis to detect deletions, conventional PCR methods are not suitable. Therefore, the information on the breakpoints allows us to detect the heterozygous deletion using conventional PCR methods.

We also found a novel point mutation in exon 6 of *PINK1*. The deletional mutation and the point mutation were located in the putative serine/threonine kinase domain of *PINK1*. Mutations in this region of *PINK1* may be important for the pathogenesis, as loss of function at this domain may affect the kinase activity or substrate recognition.[5]

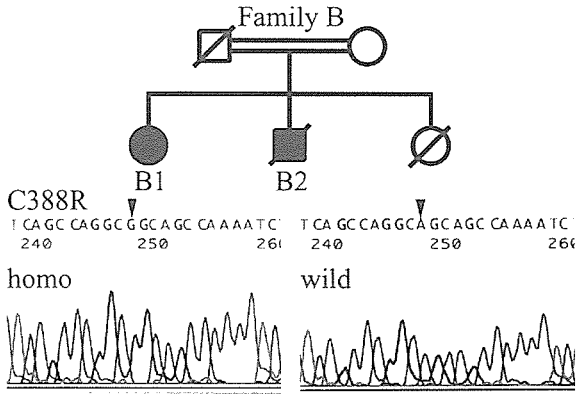

Figure 2. Pedigree and chromatograms of Family B illustrate missense mutation in a brother and a sister. A homozygous missense mutation (C388R) was found in exon 6. Complementary sequences are presented for exon 6.

Table *Clinical features of the three patients with* PINK1 *mutation*

	PINK1 mutation		
	13516-18118del homozygous	C388R homozygous	C388R homozygous
	Patient A1	Patient B1	Patient B2
Country	Japan	Japan	Japan
Consanguinity	−	+	+
Age at onset, y	38	39	44
Disease duration, y	25	16	5
Sex	F	F	M
Resting tremor	+	+	−
Rigidity	−	+	+
Bradykinesia	+	+	+
Postural instability	+	+	+
Frozen gait	+	+	−
Clinical response to levodopa	+	+	+
Wearing off	+	−	−
"On"/"off"	+	−	−
Asymmetry at onset	+	+	Not clear
Incontinence	−	−	−
Urinary urgency	−	−	−
Levodopa-induced dyskinesia	−	+	−
Sleep benefit	+	−	+
Dystonia at onset	+	−	−
Hyperreflexia	+	+	+
Dementia	+	−	−
Depression	+	−	−
Hallucination	+	−	−
Other psychosis	−	−	−

Young age at onset, parkinsonism, and good response to levodopa were noted in all three patients.

Because it is difficult to distinguish *PINK1*-positive ARPD from the *PINK1*-negative one,[8] a genetic approach is required for accurate diagnosis. In this study, the clinical manifestations of these three patients almost resembled those of patients with *parkin* mutations, although some features were different. For example, the age at onset was a little later than that of patients with *parkin* mutations. Patients B1 and B2 showed lack of dystonia at onset. Adding to our previous study, two of the 12 (17%) patients with PD with a *PINK1* point mutation (E417G and Q239X/R492X) showed dystonia at onset.[8,10] In a previous study of 101 patients with *parkin* mutation, dystonia at onset was noted in 42% of the patients, while dystonia was noted in 22% of the 85 patients without *parkin* mutation.[6] The lack of dystonia might help us to distinguish *PINK1*-positive ARPD from *parkin*- or *DJ-1*–positive ARPD.[8] In addition to dystonia, Patient A1 developed dementia. In contrast, patients with *parkin* mutations rarely develop dementia. Taken together with our previous study, the frequency of dementia in patients with *PINK1* mutations was 15.4% (2/13). Another patient who developed dementia had a nonsense mutation (R246X) in exon 3.[8,10] No patients with missense *PINK1* mutations had dementia so far.[8,10] Thus, in addition to the deletional mutation described in the present study, the defect of the putative serine/threonine kinase domain, including the 3'-terminal of *PINK1*, may be related to a more severe disease compared with missense mutations. However, further studies are needed to make any definite conclusion on the genetic-clinical correlation.

By combining our previous study, *PINK1* mutations were found in eight of 90 (8.9%) *parkin*- and *DJ-1*-negative ARPD families.[8,10] *PINK1* mutations appear to be the second most common in ARPD after *parkin*. However, the frequency of the mutation is not high enough to account for the majority of *parkin*-negative ARPD, and our results indicate that as many as 40% of our families were negative for *parkin*, *PINK1*, and *DJ-1*.

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Urinary 8-hydroxydeoxyguanosine levels as a biomarker for progression of Parkinson disease

Abstract—8-Hydroxydeoxyguanosine (8-OHdG) has been used to evaluate oxidative stress. The authors investigated urinary 8-OHdG levels in 72 patients with Parkinson disease (PD) and in normal and disease control groups. The mean urinary 8-OHdG increased with the stage of PD and was not influenced by the current dose of DOPA. Our results suggest that urinary 8-OHdG is a potentially useful biomarker for evaluating the progression of PD.

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Oxidative damage to DNA is thought to be involved in aging and various diseases. Reactive oxygen species (ROS) including hydroxyl radical and H_2O_2 react with guanine residues in DNA and produce 8-hydroxydeoxyguanosine (8-OHdG). Conversely, oxidized DNAs are continuously repaired to prevent mutagenesis, and the excised 8-OHdG is excreted in the urine, which is considered a key biomarker of oxidative DNA damage.

Although the primary cause of Parkinson disease (PD) is still unknown, oxidative stress and mitochondrial respiratory failure are implicated in the loss of dopaminergic neurons. Indeed, several lines of evidence implicate enhanced oxidative stress in the pathogenesis of PD, dopamine being one of the main candidates. The beneficial effects of levodopa in the treatment of PD are beyond doubt; however, the controversy about its accelerating effects on the neurotoxic process is still under discussion.

Recently, urinary levels of 8-OHdG were measured in patients with cancer and diabetes mellitus to evaluate the clinical stage or the response to therapy.[1,2] In PD, there is an apparent selective increase in 8-OHdG levels in the substantia nigra.[3] Moreover, the 8-OHdG levels in serum and CSF are increased in these patients.[4] Based on this background, we postulated that if positive, urinary 8-OHdG could be a good biomarker for PD.

Methods. We studied 72 patients with PD (mean age 67.3 ± 1.6 years, ± SEM, range 47 to 88), 16 patients with multiple system atrophy (MSA) (age 63.7 ± 1.7 years, range 51 to 79), and 48 normal controls (age 57.5 ± 0.6 years, range 41 to 85). Diagnosis of MSA was based on the criteria of Gilman et al.[5] and that of PD on those of Calne et al.[6] PD was classified into five stages[7] (table). Smokers and obese subjects were excluded from the study because these factors influence urinary 8-OHdG. Except for one patient, patients with MSA could not walk alone and were classified as stage V of Yahr classification. The mean disease duration in MSA patients was 7.8 ± 1.4 years. The study protocol was approved by the Human Ethics Review Committee of Juntendo University School of Medicine.

Urinary 8-OHdG concentrations were measured using an ELISA using a monoclonal antibody specific for 8-OHdG (Nippon Yushi, Tokyo, Japan). Urine samples were obtained from each individual in the morning (between 9 to 12 AM) and immediately stored at −80 °C. Patients and controls avoided physical activity in the last 24 hours before urine sampling. In hospitalized patients with an indwelling urinary catheter, we clamped the catheter in the morning and collected urine samples after a fixed period of time. Urine samples were centrifuged at 1000*g* for 15 minutes at 4 °C and the supernatant was used for 8-OHdG measurement. ELISA was carried out in triplicate and in a blinded fashion, and the average value was used for statistical analysis. The sensitivity of ELISA ranged from 0.5 to 200 ($\times 10^{-6}$ mg/dL). We also measured urinary creatinine (mg/dL) and 8-OHdG values were expressed relative to urinary creatinine [urinary 8-OHdG/creatinine] ($\times 10^{-6}$) to adjust for muscle mass. For statistical analysis, the Student *t*-test was used for paired comparisons. All data were expressed as mean ± SEM. A p value less than 0.05 denoted a significant difference.

Results. Urinary 8-OHdG/creatinine ratio ranged from 6.61 to 23.18 in normal controls, 11.90 to 68.69 in PD, and 7.76 to 30.81 in MSA. The mean ratio of patients with PD was higher than that of age-matched control subjects ($p < 0.01$) and patients with MSA ($p < 0.05$, figure 1B. The ratio correlated with age in normal subjects ($r = 0.61$, $p < 0.01$; figure 2A but not in patients with PD ($r = 0.37$, $p = 0.62$; figure 2B.

The urinary 8-OHdG/creatinine ratio for each PD stage was Stage I: 15.0 ± 1.1 (range 11.90 to 23.02), Stage II: 22.9 ± 1.8 (range 13.47 to 32.00), Stage III: 30.2 ± 3.2 (range 20.53 to 50.11), Stage IV: 36.9 ± 2.3 (range 26.72 to 51.00), and Stage V: 46.6 ± 2.0 (range 39.76 to 68.69). The ratio increased significantly with the progression of the disease ($p < 0.01$, for Stages I and II, Stages II and IV, and Stages IV and V; figure 1A. There was no correlation between the current dose of levodopa and urinary 8-OHdG/creatinine ratio ($r = 0.05$, $p = 0.70$; figure 1C.

Discussion. Oxidative DNA damage is implicated in both aging and PD. Our results showed a significant correlation between urinary 8-OHdG excretion and the normal aging process. However, no such relationship was noted in patients with PD. These results suggest the involvement of other causes of oxidative damage in patients with PD.

Our results showed high urinary 8-OHdG concentrations in patients with PD and that these levels increased with the progression of the disease. It is noteworthy that the mean level in Stage I was not different from that of the normal controls ($p > 0.05$) and the levels in Stages I and II were not different from those of MSA. Thus, this biomarker is not suitable for early diagnosis of the disease. The cutoff

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Table *Clinical characteristics of patients with Parkinson disease and patients with MSA*

	Parkinson disease stage						MSA
	I	II	III	IV	V	Total	total
n	13	16	18	14	11	72	16
Sex, M/F	4/9	6/10	8/10	6/8	4/7	28/44	6/10
Age, y	60.5	62.8	66.2	73.9	75.5	67.3	63.7
Duration of disease, y	5.7	7.2	10.0	11.3	14.1	9.4	7.8
Levodopa, mg/d	350	422	456	571	350	435	325

MSA = multiple system atrophy.

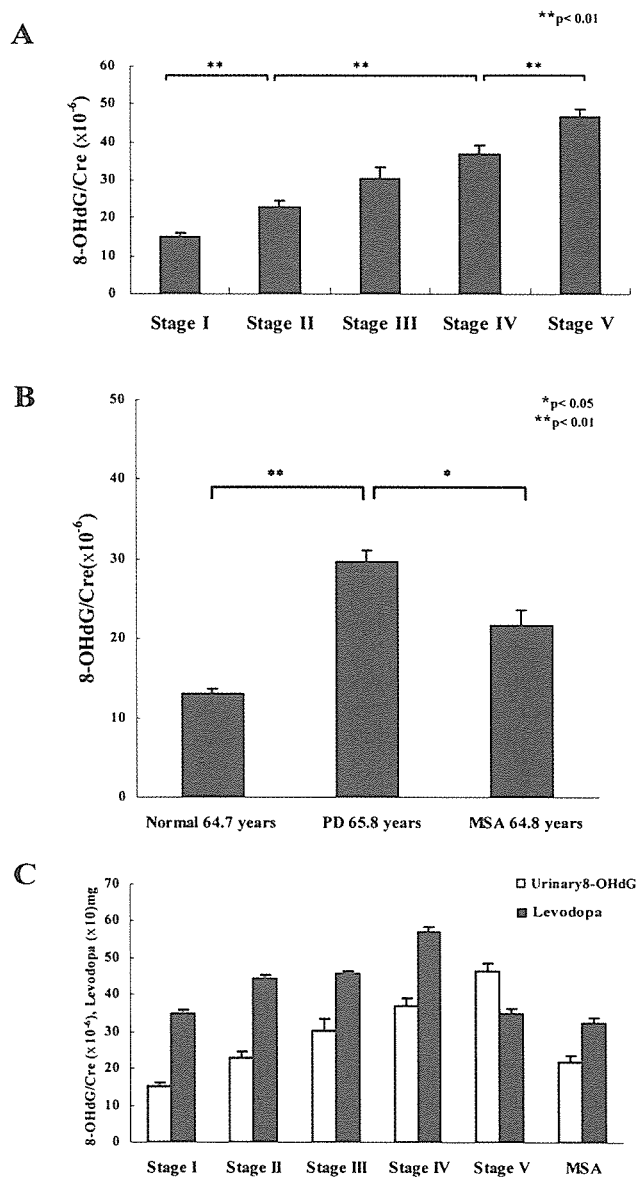

*Figure 1. (A) The mean urinary 8-hydroxydeoxyguanosine (8-OHdG)/creatinine ratio increased with the stage of Parkinson disease (PD). (B) The mean ratio was significantly higher in PD than in age-matched controls and patients with multiple system atrophy. (C) The current dose of levodopa did not influence the urinary 8-OHdG/creatinine ratio (r = 0.05, p = 0.70). Data are mean ± SEM. *p < 0.05, **p < 0.01.*

ratios were 23.18 for normal controls and 30.81 for patients with MSA. The ratio exceeded 30.81 only in patients with PD. High 8-OHdG levels in PD may be due to parkinsonism including rigidity. Rigidity may be viewed as a physical exercise load. In this regard, previous studies showed that urinary 8-OHdG levels in physically trained individuals were not different from healthy control subjects and suggested that exercise training may enhance antioxidant defense mechanisms in human skeletal muscles. It is possible that the level of systemic physical stress in patients with PD may exceed the level that could be handled by the defense mechanisms compared with continuous physical exercise.

Another potential mechanism for the increased 8-OHdG in PD is systemic mitochondrial failure[8] including brain, platelets, and skeletal muscles. Mitochondria are the most important intracellular source of ROS. In particular, systemic mitochondrial dys-

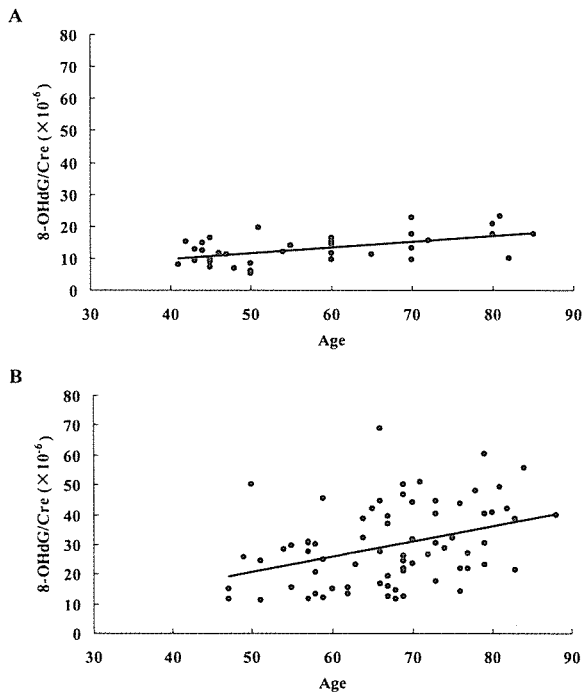

Figure 2. Urinary 8-hydroxydeoxyguanosine/creatinine (Cre) ratio of normal subjects (r = 0.61, p < 0.01) (A) and patients with Parkinson disease (r = 0.37, p = 0.62) (B).

function is considered in PD. In this regard, an apparent selective increase in 8-OHdG levels in the substantia nigra was reported. However, the characteristically high levels of the urinary 8-OHdG/creatinine ratio in our patients suggest that the mitochondrial disturbance is not solely limited to the brain but also spread over other organs such as skeletal muscles and platelets. In fact, we found no increase in 8-OHdG levels in patients without PD with cerebral thrombosis or embolism vs normal controls ($p > 0.05$ data not shown). Thus, it is unlikely that the source of high urinary 8-OHdG is the brain. The most likely organ responsible for increased 8-OHdG is skeletal muscle because the muscle tissue largely depends on an efficient oxidative energy metabolism. Previous studies reported mitochondrial dysfunction in skeletal muscles in PD.[9] We postulate that urinary 8-OHdG in PD reflects increased systemic levels of oxidative DNA damage in skeletal muscles.

Previous studies also evaluated 8-OHdG levels in various biological samples of PD patients. Mean concentrations of 8-OHdG in the serum and CSF were significantly high in PD, but the CSF concentration was generally much lower than expected considering serum 8-OHdG concentrations.[4] In this regard, it is possible that the presence of serum proteins could result in overestimation of serum 8-OHdG. Furthermore, others showed higher lipoprotein oxidation in plasma and CSF applying the lipoprotein kinetics curve.[10] However, our assay with ELISA using urinary samples allows easier evaluation of the oxidative status, and it is simple and noninvasive. We evaluate 8-OHdG levels in various stages of the disease.

Analysis of the relationship between levodopa and oxidative damage showed no correlation between 8-OHdG and the mean current dose of levodopa. Dopamine is metabolized by monoamine oxidase to generate reactive and toxic hydroxyl radicals. Our data suggest that oxidative stress is not dependent on the current dose of levodopa. Considering that 8-OHdG levels correlated with Hoehn and Yahr stage of the disease, it is possible that cumulative exposure to levodopa could influence PD progression. However, the systemic neurotoxic effects of levodopa are still not clear at present.

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Geographic and ethnic differences in frequencies of two polymorphisms (D/N394 and L/I272) of the *parkin* gene in sporadic Parkinson's disease

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Abstract

In this report, we evaluated the allele frequency of the D/N394 single nucleotide polymorphism (SNP) in exon 11 of the *parkin* gene in 200 Japanese patients with sporadic Parkinson's disease (PD) and 200 normal controls. Although the reported allele frequency of G-to-A (D/N394) is 2% in Caucasians, this SNP was not detected in Japanese patients and healthy controls. Evaluation of L/I272 polymorphism, a C-to-A transition in exon 7, showed the polymorphism in only six controls, but not in PD patients. Our results suggest that the frequencies of *parkin* polymorphisms are different among Asians and Caucasians.

Keywords: Allele frequency; TaqMan assay; Restriction fragment length polymorphism; Geographic distribution; Ethnicity; Race

1. Introduction

Parkinson's disease (PD) is the second most common neurodegenerative disorder, next to Alzheimer's disease. The primary cause of PD is unknown, however, mitochondrial failure and oxidative stress are implicated in its pathogenesis [1,2]. There is general agreement that the interaction of genetic factors and environmental factors are involved in the pathogenesis of PD.

Parkin is the causative gene of early onset parkinsonism, which is characterized pathologically by neurodegeneration of dopaminergic neurons and gliosis in the substantial nigra, without Lewy body formation [3]. About 50% of cases with autosomal recessive early onset PD (AREO-PD) including autosomal recessive juvenile parkinsonism (AR-JP) cases and 18% of early onset (<40 years) sporadic PD patients were found to have mutations in the *parkin* gene [4]. Most of the *parkin* mutations are consistent with autosomal-recessive mutation [5–8], however, at times PD patients have a single *parkin* allele mutation suggestive of a dominant effect or a haplo-insufficiency [9]; such mutations might act as a risk factor for sporadic PD.

At least four polymorphisms of the *parkin* gene have been reported so far. Firstly, we reported three polymorphisms including S/N167, R/W366, and V/L380 [10]. Among them, the frequency of S/N167 in Asian population is different from that in Caucasians [10-20]—suggesting geographic and/or racial differences in the frequencies of *parkin* gene polymorphisms. Thus, it is important to determine the frequency of the *parkin* gene polymorphisms in different races. Here, we report the allele frequencies of L/I272 in exon 7 and D/N394 in exon 11 of the *parkin* gene in Japanese patients with sporadic PD since no information on the allelic frequency of both SNPs is currently available. In addition, we compare the frequencies of the *parkin* gene polymorphisms between Asians and Caucasians based on the results of the present study and those published by other investigators [10-20].

2. Subjects and methods

2.1. Subjects

The subjects of this study were 200 (82 males and 118 females) patients with sporadic PD and 200 (101 males and 99 females) control subjects free of neurodegenerative disorders. The recruited patients were from the Department of Neurology at Juntendo University School of Medicine,

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Tokyo. Some of the control subjects were the spouses of the PD patients studied. The diagnosis of PD was based on international criteria [21] and included the presence of two or more cardinal clinical features of the disease (e.g. bradykinesia, resting tremor, cogwheel rigidity, and postural reflex impairment). The onset of the disease in all PD patients studied was after the age of 40 years. The mean ± SD age at the onset of the disease was 56.7 ± 10.7 years. The mean age of control subjects was 58.3 ± 16.3 years. The study was approved by the ethics review committee of Juntendo University.

2.2. Methods

2.2.1. DNA isolation

Human genomic DNA as a template for polymerase chain reaction (PCR) was isolated from peripheral blood leukocytes according to the standard methods using a QIAamp DNA Blood Maxi Kit (50) (Qiagen, Hilden, Germany) as described previously [22]. Samples were used as soon as possible and stored at 4 °C until analysis.

2.2.2. Detection of D/N394 using TaqMan assay

We identified the polymorphism of D/N394 in the *parkin* gene using the TaqMan assay for single nucleotide polymorphism (SNP), which included two TaqMan minor groove binder (MGB) probes and two primers (forward and reverse) along with the probes. In contrast, polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) was performed in all the studies that were reported previously [10–13,15–17,19–20]. The probes and primers for allelic discrimination assays were designed by Applied Biosystem (Foster City, CA). Allelic discrimination of *parkin* D/N 394 polymorphism was performed by TaqMan assay using the ABI PRISM 7700 Sequence Detection System. The PCR reactions were carried out on 96-well plates. The SNP analyzed by the TaqMan assay was PCR amplified from genome DNA. All PCR reactions were run in triplicate, and contained about 100 ng of patient DNA, 12.5 μl of TaqMan Universal PCR Master and 0.625 μl of Allelic Discrimination Mixture. The latter consisted of 10 μM Forward primer, 10 μM Reverse primer, 5 μM TaqMan (FAM) probe, 5 μM TaqMan (VIC) probe, and Milli Q water 10.875 μl, template 1 μl, with a total volume of 25 μl. The forward primer sequences used were 5′-AACGCCTTTCCTCTTTGTTTCC-3′, the reverse primer sequences used were 5′-GAGGCTGCTTCC-CAACGA-3′, the probe sequences of FAM used were 5′-ACAGAGTCAATGAAAG-3′, the probe sequences of VIC used were 5′-AGAGTCGATGAAAGAG-3′. Appropriate negative controls were also run. TaqMan assay was performed on an ABI Prism 7700 Sequence Detection System (SDS, PE Biosystems). This reaction with allelic discrimination assays was used under the following conditions: 50 °C for 2 min, 95 °C for 10 min, and then 35 cycles of amplification (92 °C denaturation for 15 s, and annealing/extension for 60 s at 60 °C).

After placing the PCR amplification plates in the analysis plate reader, the fluorescence data were analyzed and genotypes were classified with the post-PCR read analysis for allelic discrimination. Furthermore, we compared the frequencies of the four alleles with one another relative to the previously reported *parkin* polymorphisms [10–20].

2.2.3. Detection of L/I272 using PCR-RFLP method

Exon 7 of *parkin* gene was amplified by PCR using two primer pairs (forward primer of L/I272 were 5′-TGCTGCCTTTCCACACTGAC-3′, reverse primer of L/I272 were 5′-CATGCTAGACTTACCCACAC-3′). PCR contained template DNA 1.0 μl (100 ng), 10× PCR buffer 5.0 μl, 2 mM dNTP mixture 1.0 μl, 10 μM Sense Primer (L/I272-S) 1.0 μl, 10 μM Antisense Primer (L/I272-AS) 1.0 μl, AmpliTaq Gold 0.5 μl, and Milli Q 40.5 μl. The PCR conditions for L/I272 in exon 7 amplification were as follows: initial denaturation at 94 °C for 10 min was followed by 40 cycles of denaturation at 94 °C for 30 s, annealing at 57 °C for 30 s, and extension at 72 °C for 30 s, with a final extension at 72 °C for 10 min. The polymorphism at amino acid position 272 in exon 7 was analyzed by digestion with *Tfi*I (New England BioLabs Inc.). The L/I272 polymorphism allele created a restriction site for *Tfi*I. Restriction Enzyme Digestion contained 10 μl of PCR products, 2.0 μl of 10× NEB Buffer 3, 0.5 μl of *Tfi*I (5 units/μl), and 7.5 μl of Milli Q. The reaction lasted 4 h at 65 °C. Finally, 10 μl of the reaction product was applied for 2% agarose gel electrophoresis and stained with ethidium bromide.

2.2.4. Statistical analysis

Statistical Analysis was performed using StatView-J version 4.02 (Abacus Concept, SAS, Inc., San Francisco, CA) employing the χ^2-test and Fisher's exact probability. In addition, we compared the frequencies of the *parkin* gene polymorphisms among different ethnic populations based on the results of previous studies [10–22]. Finally, we compared the frequencies of each SNPs between Asians and Caucasians using the data reported in the literature [10–20].

3. Results

Regarding SNP analysis on D/N394, the frequency of allele G was 100%, in both PD patients and the control subjects (Table 1). We also confirmed the polymorphisms of D/N394 in the *parkin* gene using the ABI PRISM 7700 Sequence Detection System and TaqMan assay (Fig. 1). These results indicated no differences in the genotypes in SNP of D/N394 between Japanese PD patients and the control subjects. Regarding the L/I272 polymorphism, all PD patients and 98.5% of the control subjects had allele C; only six normal control subjects had allele A in

Table 1
Allele frequencies of D/N394 and L/I272 polymorphisms in *parkin* in sporadic PD and normal control subjects

	Control (%)	PD (%)	Total (%)
Number of subjects	200	200	400
Number of chromosomes	400	400	800
D/N394 polymorphism			
Allele frequency			
Allele G	400 (100%)	400 (100%)	800 (100%)
Allele A	0 (0%)	0 (0%)	0 (0%)
L/I272 polymorphism			
Allele frequency			
Allele C	394 (98.5%)	400 (100%)	794 (99.2%)
Allele A	6 (1.5%)	0 (0%)	6 (0.8%)

There were significant differences in allele frequencies of L/I272 polymorphism in *parkin* between patients with Parkinson's disease (PD) and controls ($\chi^2=6.045$, df = 1, $p=0.0139<0.05$).

heterozygous state (Table 1 and Fig. 3A) ($\chi^2=6.045$, df = 1, $p=0.0139<0.05$). The results of RFLP on 2% agarose gel showed L/I272 polymorphism. The wild type allele was differentiated from the mutant allele after digestion with *Tfi*I (Fig. 2). We compared the frequencies of four alleles of the *parkin* polymorphisms (S/N167, R/W366, V/L380, and D/N394) with the frequencies reported in previous studies [10–20] (Fig. 3B–E, Table 2). In addition, the frequencies of the SNPs including S/N167, R/W366, V/L380, and D/N394 were significantly different between Asians and Caucasians (Table 3). Among them, the frequency of S/N167 in Asian population was higher than in Caucasians. The frequency of R/W366 polymorphism in Asian population was slightly higher than in Caucasians. In contrast, the frequencies of V/L380 and D/N394 were higher in Caucasians than in Asians.

4. Discussion

In the present study, we analyzed the frequencies of two polymorphisms of the *parkin* gene in 200 patients with sporadic PD and 200 normal control subjects. We found no statistically significant differences in the genotype and allele distribution of the D/N394 in exon 11. Our study does not provide direct evidence that *parkin* could be interrelated to sporadic PD either as a risk factor or protective factor.

Our results showed polymorphism of L/I272 in six controls, suggesting that this polymorphism could be protective against the development of PD although the frequency is too small to make a firm conclusion. Its frequency has not yet been reported in various populations. Recently, Takao et al. [23] reported the results of autopsy examination of a patient with L/I272 who showed diffuse Lewy body disease. They reported also that the L/I272 polymorphism was detected in a normal Japanese control. Therefore, this replacement cannot be considered pathogenic but rather a rare polymorphism.

A cross comparison of allele frequencies of G-to-A transition in exon 4 (S/N167) in *parkin* between Japanese and other ethnic groups showed that the Japanese

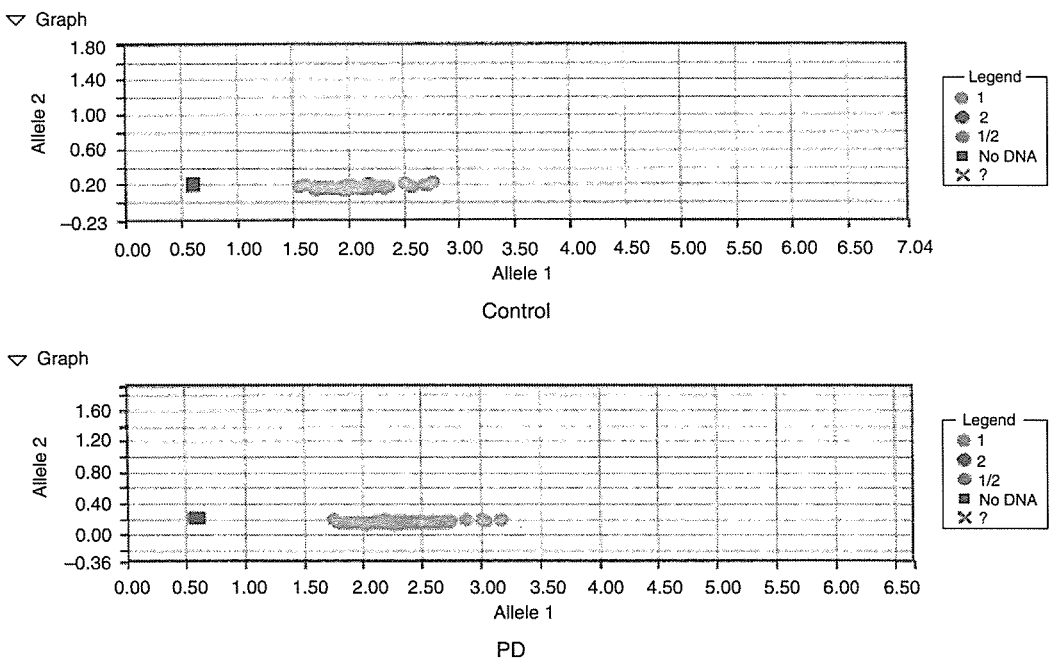

Fig. 1. Results of D/N394 polymorphism in the *parkin* gene using ABI PRISM 7700 Sequence Detection System and TaqMan assay. Cleavage separates the reporter dye from the quencher dye, which results in increased fluorescence by the reporter dye. Allele 1 homozygote, allele 2 homozygote, and allele 1 and 2 heterozygote are separated by reporter fluorescence. Red circles indicate allele 1 homozygote, blue circles indicate allele 2 homozygote, green circles indicate alleles 1 and 2 heterozygote, and the black square indicates no template control. (For interpretation of the reference to colour in this legend, the reader is referred to the web version of this article.)

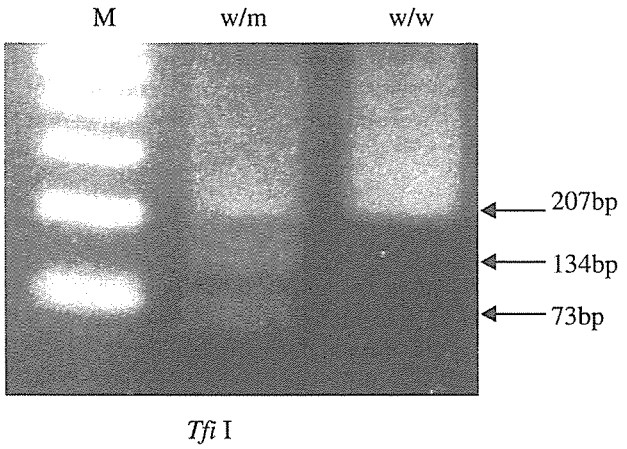

Fig. 2. Results of restriction fragment length polymorphism (RFLP) on 2% agarose gel of L/I272 polymorphism in the *parkin* gene. *Tfi*I RFLP of polymerase chain reaction (PCR) of exon 7 on 2% agarose gel. M is a 100-base pair ladder marker; w/m and w/w indicate heterozygote and wild-type homozygote, respectively.

(Wang et al. [10]; Satoh et al. [11]) were similar to the Chinese PD (China [14]; Taiwan [15]), but the frequencies in these two populations were higher than in those reported in studies from Italy [18], Spain [17], North America [13], Europe [19,20], and Finland [16]. Furthermore, the R/W366 was found in Japan [10], and China (Taiwan) [15], but not in Finland [16] or Italy [18]. The allele frequencies of V/L380 in *parkin* reported in studies from Finland [16], Italy [18], USA [13] and Europe [10–20] were higher than in studies from Japan [10] and China [15] in patients with PD. The D/N394 (G-to-A) in exon 11 has also been found in North America [13], Spain [17], Italy [18], and other European countries [10–20]. In the present study, this polymorphism (D/N394) was not found in Japanese patients with sporadic PD and healthy controls, suggesting that this polymorphism is very rare. The frequency of S/N167 polymorphism was higher in Asians than in Caucasians. In contrast, the V/L380 polymorphism was higher in Caucasians than in Japanese. Considering the racial and geographic distribution, the S/N167 polymorphism seems to be much older than the V/L380 polymorphism.

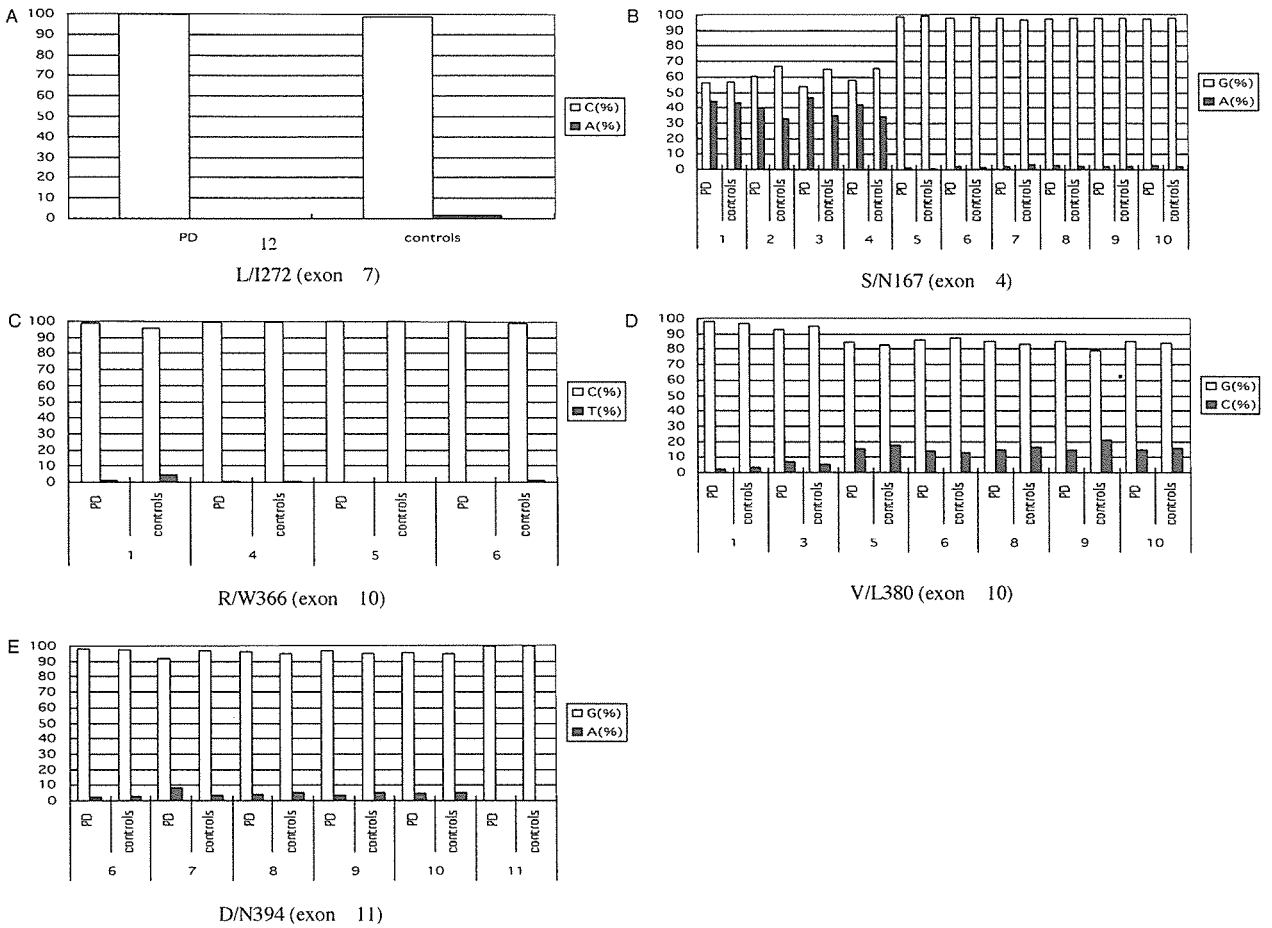

Fig. 3. *Parkin* polymorphisms and frequencies of various alleles in various countries. (A) *Parkin* polymorphism of L/I272; (B) *Parkin* polymorphism of S/N167; (C) *Parkin* polymorphism of R/W366; (D) *Parkin* polymorphism of V/L380; (E) *Parkin* polymorphism of D/N394. 1, Japan (Ref. [13]); 2, Japan (Ref. [12]); 3, China (Ref. [16]); 4, Taiwan (Ref. [17]); 5, Finland (Ref. [18]); 6, Italy (Ref. [20]); 7, Spain (Ref. [19]); 8, North America (Ref. [15]); 9, European1 (Ref. [21]); 10, European2 (Ref. [22]); 11, Japan (this study about D/N394); 12, Japan (this study about L/I272).

Table 2
Allele frequencies of S/N167, R/W366, V/L380 and D/N394 polymorphisms in *parkin* in patients with sporadic PD (SPD) and normal control subjects in different countries

		Japan (Wang)	Japan (Satoh)	China	China (Taiwan)	Finland	Italy	Spain	North America	Europe1	Europe2
S/N167 polymorphism											
Allele G (%)											
	SPD	56.6	60.6	53.4	58.2	99.0	97.9	98.0	97.5	97.9	97.5
	Control	56.3	66.9	64.9	65.8	99.5	98.5	97.0	98.4	98.0	97.9
Allele A (%)											
	SPD	43.4	39.4	46.6	41.8	1.0	2.1	2.0	2.5	2.1	2.5
	Control	43.7	33.1	35.1	34.2	0.5	1.5	3.0	1.6	2.0	2.1
Genotype GG (%)											
	SPD	36.9	29.6	24.1		98.0	95.8	95.3	94.9	96.1	
	Control	36.3	44.0	41.1		99.0	97.0	94.7	96.9	96.0	
Genotype GA (%)											
	SPD	39.4	62.0	58.6		2.0	4.2	4.7	5.1	3.9	
	Control	40.0	45.9	47.6		1.0	3.0	5.3	3.1	4.0	
Genotype AA (%)											
	SPD	23.7	8.4	17.3		0	0	0	0	0	
	Control	23.7	10.1	11.3		0	0	0	0	0	
P-value		0.9365	0.23	0.0903	0.1232	0.7111	0.6527	0.8359	0.4532	0.9762	0.5309
R/W366 polymorphism											
Allele C (%)											
	SPD	98.8			99.5	100	100				
	Control	95.7			99.5	100	99.0				
Allele T (%)											
	SPD	1.2			0.5	0	0				
	Control	4.3			0.5	0	1.0				
Genotype CC (%)											
	SPD	97.5				100	100				
	Control	91.3				100	98.0				
Genotype CT (%)											
	SPD	2.5				0	0				
	Control	8.7				0	2.0				
Genotype TT (%)											
	SPD	0				0	0				
	Control	0				0	0				
P-value		0.0168			0.9774		0.1670				
V/L380 polymorphism											
Allele G (%)											
	SPD	98.1			92.9	85.0	86.3		85.5	85.6	85.1
	Control	96.6			94.9	83.0	87.5		83.4	78.8	84.1
Allele C (%)											
	SPD	1.9			7.1	15.0	13.7		14.5	14.4	14.9
	Control	3.4			5.1	18.0	12.5		16.6	21.2	15.9
Genotype GG (%)											
	SPD	96.3				72.1	75.8		72.4	77.5	
	Control	93.1				68.6	77.0		75.2	62.4	
Genotype GC (%)											
	SPD	3.7				25.8	21.1		25.5	21.6	
	Control	6.9				27.7	21.1		21.8	32.8	
Genotype CC (%)											
	SPD	0				2.0	3.1		2.1	0.9	
	Control	0				3.7	2.0		3.0	4.8	
P-value		0.2190			0.4585	0.4094	0.7288		0.7271	0.0077	0.5198
D/N394 polymorphism											
Allele G (%)											
	SPD						98.15	92.0	96.4	96.6	95.3
	Control						97.5	97.0	94.9	95.2	95.1

(*continued on next page*)

Table 2 (*continued*)

	Japan (Wang)	Japan (Satoh)	China	China (Taiwan)	Finland	Italy	Spain	North America	Europe1	Europe2
Allele A (%)										
SPD						1.85	8.0	3.6	3.4	4.7
Control						2.5	3.0	5.1	4.8	4.9
Genotype GG (%)										
SPD						93.7	85.9	91.3	93.1	
Control						95.0	95.3	94.4	90.4	
Genotype GA (%)										
SPD						6.3	12.9	7.5	6.9	
Control						5.0	4.7	5.6	9.6	
Genotype AA (%)										
SPD						0	1.2	1.3	0	
Control						0	0	0	0	
P-value						0.1540	0.0029	0.2240	0.4688	0.8604

Japan Wang (Ref. [13]); Japan Satoh (Ref. [12]); China (Ref. [16]); China Taiwan (Ref. [17]); Finland (Ref. [18]); Italy (Ref. [20]); Spain (Ref. [19]); North America (Ref. [15]); Europe1 (Ref. [21]); Europe2 (Ref. [22]).

Table 3
Allele frequencies of V/L380 polymorphism in *parkin* in Asians and Caucasians

	Asian (%)	Caucasians (%)	Total (%)
S/N167 polymorphism			
Number of subjects	568	1335	1903
Number of chromosomes	1136	2670	3806
Allele frequency			
Allele G	646 (56.9%)	2621 (98.2%)	3267 (85.8%)
Allele A	490 (43.1%)	49 (0.8%)	539 (14.2%)
R/W366 polymorphism			
Number of subjects	320	195	515
Number of chromosomes	640	390	1030
Allele frequency			
Allele C	622 (97.2%)	388 (9.5%)	1010 (98.1%)
Allele T	18 (2.8%)	2 (0.5%)	20 (1.9%)
V/L380 polymorphism			
Number of subjects	320	1039	1359
Number of chromosomes	640	2078	2718
Allele frequency			
Allele G	623 (97.3%)	1890 (91.0%)	2513 (92.5%)
Allele C	17 (2.7%)	188 (9.0%)	205 (7.5%)
D/N394 polymorphism			
Number of subjects	400	668	1068
Number of chromosomes	800	1336	2136
Allele frequency			
Allele G	800 (100%)	1275 (95.4%)	2075 (97.1%)
Allele A	0 (0%)	61 (4.6%)	61 (2.9%)

There were significant differences in allele frequencies of S/N167 polymorphism in *parkin* between Asians and Caucasians ($\chi^2 = 1118.127$, df = 1, $p < 0.0001$). There were significant differences in allele frequencies of R/W366 polymorphism in *parkin* between Asians and Caucasians ($\chi^2 = 6.731$, df = 1, $p = 0.0095 < 0.01$). There were significant differences in allele frequencies of V/L380 polymorphism in *parkin* between Asians and Caucasians ($\chi^2 = 28.659$, df = 1, $p < 0.0001$). There were significant differences in allele frequencies of D/N394 polymorphism in *parkin* between Asians and Caucasians ($\chi^2 = 37.601$, df = 1, $p < 0.0001$).

The above results and those of previous studies [10–20] suggest ethnic differences in allele frequencies. It is important to identify the differences in the frequencies among various populations, because single nucleotide polymorphism may not only be involved in the pathogenesis of the disease as a risk or a protective factor, but could also explain the geographic distribution.

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A Combinatorial Code for the Interaction of α-Synuclein with Membranes*

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Considerable genetic and pathological evidence has implicated the small, soluble protein α-synuclein in the pathogenesis of familial and sporadic forms of Parkinsons disease (PD). However, the precise role of α-synuclein in the disease process as well as its normal function remain poorly understood. We recently found that an interaction with lipid rafts is crucial for the normal, pre-synaptic localization of α-synuclein. To understand how α-synuclein interacts with lipid rafts, we have now developed an *in vitro* binding assay to rafts purified from native membranes. Recapitulating the specificity observed *in vivo*, recombinant wild type but not PD-associated A30P mutant α-synuclein binds to lipid rafts isolated from cultured cells and purified synaptic vesicles. Proteolytic digestion of the rafts does not disrupt the binding of α-synuclein, indicating an interaction with lipid rather than protein components of these membranes. We have also found that α-synuclein binds directly to artificial membranes whose lipid composition mimics that of lipid rafts. The binding of α-synuclein to these raft-like liposomes requires acidic phospholipids, with a preference for phosphatidylserine (PS). Interestingly, a variety of synthetic PS with defined acyl chains do not support binding when used individually. Rather, the interaction with α-synuclein requires a combination of PS with oleic (18:1) and polyunsaturated (either 20:4 or 22:6) fatty acyl chains, suggesting a role for phase separation within the membrane. Furthermore, α-synuclein binds with higher affinity to artificial membranes with the PS head group on the polyunsaturated fatty acyl chain rather than on the oleoyl side chain, indicating a stringent combinatorial code for the interaction of α-synuclein with membranes.

Recent work has indicated an important role for the protein α-synuclein in the pathogenesis of Parkinsons disease (PD).[1] Mutations in α-synuclein produce a highly penetrant but rare autosomal dominant form of PD (1–3). In other families, increased dosage of the wild type gene suffices to cause PD (4). Although mutations in α-synuclein do not contribute to idiopathic PD, the brains of most patients contain abundant α-synuclein in the form of Lewy bodies and dystrophic neurites (5–7), supporting a role for the protein in sporadic forms of the disease. However, the mechanism by which α-synuclein contributes to neural degeneration remains poorly understood.

Originally identified as a synaptic vesicle-associated protein, α-synuclein has been implicated in synaptic plasticity, neurotransmitter release, and more specifically, synaptic vesicle recycling (8–12). Despite its specific localization to the nerve terminal, α-synuclein does not co-fractionate with native membranes in brain extracts, but behaves as a soluble protein (13–16). The molecular determinants that localize α-synuclein to the synapse thus remain unknown. However, α-synuclein can associate with native membranes such as axonal transport vesicles, lipid droplets produced in HeLa cells by the administration of oleic acid, and the membranes of *Saccharomyces cerevisiae* (17–19). Importantly, the A30P mutation associated with familial PD disrupts these interactions. *In vitro*, α-synuclein binds directly to artificial membranes containing acidic phospholipids in a manner that is not sensitive to the A30P mutation (20–23). The relationship of these observations to the pre-synaptic localization of α-synuclein has remained unclear.

Recently, we found that α-synuclein associates specifically with membrane microdomains known as lipid rafts (24). Lipid rafts are enriched in cholesterol, sphingomyelin, and phospholipids with saturated long chain fatty acids as well as particular proteins. Biochemically, they are defined by their insolubility in cold Triton X-100 and their low buoyant density (25). α-Synuclein expressed in cultured cells and in brain co-fractionates with detergent-resistant membranes, consistent with its raft association. Pharmacologic disruption of lipid rafts eliminates the synaptic enrichment of α-synuclein. The A30P mutation associated with familial PD also disrupts the raft association of α-synuclein, and redistributes the protein from synapses into the axon (24). Binding to lipid rafts thus contributes to the normal function of α-synuclein by localizing the protein to the nerve terminal, and may also influence its role in the pathogenesis of PD.

To define the interaction of α-synuclein with lipid rafts, we

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[1] The abbreviations used are: PD, Parkinsons disease; PS, phosphatidylserine; GST, glutathione *S*-transferase; PC, phosphatidylcholine; MES, 4-morpholineethanesulfonic acid; BB, binding buffer; DRM, detergent-resistant membrane; PK, proteinase K; CBP, calmodulin-binding peptide; SM, sphingomyelin; CHAPS, 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonic acid.

have developed an *in vitro* assay for binding to membranes prepared from native tissue. Using this assay, recombinant wild type α-synuclein binds saturably and with high affinity to lipid rafts isolated from HeLa cells and rat brain, and the A30P mutation disrupts the interaction. The assay thus faithfully recapitulates the specificity of raft association observed in cells (24). Proteolytic digestion of the raft fraction does not reduce raft association *in vitro*, indicating a direct interaction of α-synuclein with the membrane. Confirming a direct lipid interaction, α-synuclein binds to artificial membranes mimicking the composition of lipid rafts (25). Consistent with previous reports (20, 26, 27), the interaction of α-synuclein with raft-like liposomes requires phospholipids with an acidic head group, with a strong preference for phosphatidylserine (PS). In contrast to previous studies, however, our assay reveals specific requirements for the acyl chain composition of PS. A variety of synthetic PS with single defined acyl chains do not support binding of α-synuclein. Rather, binding requires a combination of PS with oleic (18:1) and polyunsaturated (20:4 and 22:6) fatty acyl chains, suggesting that a phase transition contributes to the association with lipid rafts. Furthermore, α-synuclein binds preferentially to membranes with PS on the polyunsaturated acyl chain, indicating the coordinate recognition of head group in the context of a specific side chain.

EXPERIMENTAL PROCEDURES

Materials—Antibodies to human α-synuclein (15G7), rat α-synuclein (Syn1), VGLUT1, GST, and CD55 were obtained from, respectively, Alexis Biochemicals (San Diego, CA), BD Biosciences (San Diego, CA), Chemicon (Temecula, CA), Molecular Probes (Eugene, OR), and Santa Cruz Biotechnology (Santa Cruz, CA). Secondary antibodies conjugated to horseradish peroxidase were purchased from Amersham Biosciences. Secondary antibodies conjugated to fluorescein isothiocyanate or Cy3 and Alexa 647-conjugated Annexin V were obtained from Jackson ImmunoResearch (West Grove, PA) and Molecular Probes (Eugene, OR), respectively. Cholesterol, brain sphingomyelin (SM), brain PS, egg phosphatidic acid, brain phosphatidylcholine (PC), brain phosphatidylethanolamine, 1,2-distearoyl-*sn*-glycero-3-phosphoserine (18:0 PS), 1,2-dioleoyl-*sn*-glycero-3-phosphoserine (18:1 PS), 1,2-diarachidonoyl-*sn*-glycero-3-phosphoserine (20:4 PS), and 1,2-didocosahexaenoyl-*sn*-glycero-3-phosphoserine (22:6 PS) were obtained from Avanti Polar Lipids (Alabaster, AL).

Molecular Biology and Cell Culture—The construction of α-synuclein cDNAs and purification of bacterial fusion proteins has been described previously (24). HeLa cells were grown in Dulbecco's modified Eagle's medium with 10% cosmic calf serum (HyClone, Logan, UT) at 37 °C and 5% CO_2. Dissociated hippocampal cultures containing glia were prepared from embryonic (E18.5) rats and maintained in Neurobasal medium (Invitrogen, San Diego, CA) for 2–3 weeks (28).

Preparation of Synaptic Vesicles from Rat Brain—Synaptic vesicles were prepared as previously described (29, 30). Briefly, the cortices of 200-g male Sprague-Dawley rats were homogenized in 0.32 M sucrose, 4 mM HEPES-NaOH, pH 7.4, 1 mM NaF, 1 mM Na_3VO_4, 10 μM leupeptin, 1 μM pepstatin, 1 mM phenylmethylsulfonyl fluoride, containing phosphatase inhibitor mixtures I and II (Calbiochem, La Jolla, CA) (HB). Cell debris was removed by centrifugation at 1,350 × *g* for 10 min at 4 °C, and crude synaptosomes were sedimented at 12,000 × *g* for 10 min at 4 °C. The synaptosomal pellet was washed in HB, sedimented at 13,000 × *g* for 15 min, and the resulting pellet lysed by hypo-osmotic shock in ice-cold water containing protease and phosphatase inhibitors. Lysed synaptosomes were adjusted to 9.3 mM HEPES, pH 7.4, and synaptic plasma membrane removed by centrifugation at 33,000 × *g* for 20 min. The resulting supernatant was further sedimented at 260,000 × *g* for 2 h to pellet synaptic vesicles, which were resuspended in 25 mM MES, 80 mM NaCl (binding buffer, BB) containing protease and phosphatase inhibitors as above.

Isolation of Detergent-resistant Membranes (DRMs)—DRMs were isolated from HeLa cells and synaptic vesicles as previously described (24, 31). Briefly, HeLa cells (1×10^7) or synaptic vesicles (200 μg of protein) were resuspended in 1 ml of BB containing 1% Triton X-100 and incubated on ice for 30 min with Dounce homogenization every 10 min. The resulting extract was adjusted to 42.5% sucrose, overlaid with 5 ml each of 35 and 5% sucrose in BB, and sedimented at 4 °C in a Beckman SW41 rotor at 275,000 × *g* for 18 h. Lipid rafts (250 μl) were collected at the interface between 5 and 35% sucrose, and stored at 4 °C until use.

Proteinase K (PK) Digestion—100 μl of isolated DRMs were dialyzed against 50 mM Tris-HCl, pH 8.0, 50 mM NaCl, and incubated with 10 μl of PK-agarose (Sigma) at 30 °C with rotation for the times indicated. The reaction was terminated by sedimentation of the PK-agarose. PK-treated rafts were subjected to electrophoresis through Criterion Tris-HCl polyacrylamide gels (Bio-Rad) followed by staining with the GelCode SilverSNAP Stain Kit (Pierce).

Preparation of Liposomes—Lipids of interest were mixed in chloroform, and the solvent evaporated under nitrogen. The resulting lipid film was dried under vacuum for 20 min and re-hydrated at a concentration of 2.5 mM in BB followed by vortexing. Small unilamellar vesicles were prepared by five 1-min sonication and freeze/thawing cycles, stored in the dark at 4 °C under nitrogen, and used within 1 week of production.

In Vitro Binding Assay—Recombinant α-synuclein, fused at its N terminus to the 41-residue calmodulin-binding peptide (CBP), was combined with 100 μl of DRMs or 5 μl of liposomes in BB containing 1% bovine serum albumin as nonspecific competitor, and incubated at 30 °C for 30 min. Sucrose was then added to a final concentration of 42.5%, the mixture was overlaid with 2 ml of 35% and 2 ml of 5% sucrose in BB, and sedimented at 4 °C in a Beckman SW55 rotor at 275,000 × *g* for 18 h. Ten 0.5-ml fractions were collected from the top of the gradient and either used immediately or stored at −80 °C until use, with no differences observed between unfrozen and frozen material (data not shown). Equal volumes of each fraction were separated by electrophoresis as above, electrotransferred to polyvinylidene difluoride, immunostained with appropriate antibodies, and detected using West Pico SuperSignal (Pierce). For quantitative Western blotting, protein bands were quantified using the ChemiImager System (Alpha Innotech, San Leandro, CA). Every experiment was performed independently at least twice. Unless indicated otherwise, all experiments involved the CBP fusion to α-synuclein.

Purification of Bound α-Synuclein for Mass Spectrometry—Binding was performed as above using a final concentration of 1 mM liposomes and 8 μM α-synuclein. Membrane-associated α-synuclein was collected from the 5/35% sucrose interface, solubilized in 20 mM CHAPS, the micelles were removed by centrifugation through a 10-kDa molecular mass cutoff Amicon Ultra filter (Millipore, Bedford, MA), and the buffer exchanged to 100 mM ammonium bicarbonate. For further purification, concentrated α-synuclein was separated by size exclusion chromatography on a Superose 12 column (Amersham Biosciences) in 100 mM ammonium bicarbonate at a flow rate of 0.2 ml/min. Fractions containing α-synuclein were identified by Western blotting and dried under vacuum centrifugation. Each sample was then digested overnight with 100 ng of trypsin (sequencing grade modified, Promega, Madison, WI) in 25 mM ammonium bicarbonate. Samples were analyzed by liquid chromatography-mass spectrometry using a nano-LC system (Eksigent, Livermore, CA) to separate samples for on-line analysis using a QSTAR mass spectrometer (Sciex, Concord, Ontario, Canada). The resulting data were analyzed using a combination of manual analysis and automated analysis with the locally developed Protein Prospector suite of proteomic tools (www.prospector.ucsf.edu).

Immunofluorescence—Hippocampal neurons were grown for 15–20 days *in vitro*, fixed in 4% paraformaldehyde, and immunostained for α-synuclein and VGLUT1. When indicated, Alexa 647-conjugated Annexin V was added in 2.5 mM $CaCl_2$ during the last 15 min of incubation with secondary antibodies. Fluorescent images were acquired on a Zeiss (Oberkochen, Germany) LSM 510 confocal microscope. Annexin V was pre-adsorbed by incubation with liposomes overnight at 4 °C, the bound material was removed by sedimentation at 100,000 × *g*, and the supernatant used for immunofluorescence. To allow direct comparison of Annexin V staining, images were collected using fixed laser strength, pinhole size, and detector gain.

RESULTS

α-Synuclein Binds to Purified Lipid Rafts—To understand how α-synuclein interacts with lipid rafts, we have developed an *in vitro* binding assay using recombinant α-synuclein and DRMs prepared from HeLa cells by flotation gradient (24, 31). The DRMs were incubated with recombinant α-synuclein for 30 min at 30 °C, and bound protein was separated by flotation through a second density gradient. Initially, we observed binding of both wild type and A30P α-synuclein to purified DRMs