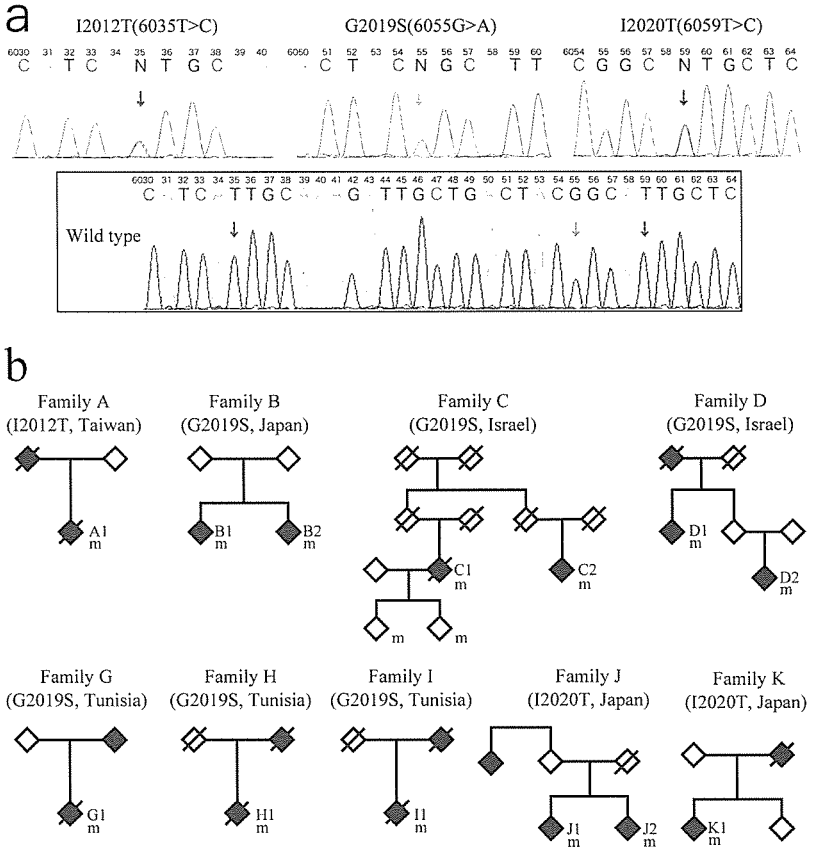

FIG. 1. a: Chromatogram showing 3 mutations in *LRRK2* exon 41. A novel 6035T>C (I2012T) mutation was found in Family A. The 6055G>A (G2019S) mutation was found in Families B-I. The 6059T>C (I2020T) mutation was found in Families J and K. **b:** Pedigree structures of Families A-D and G-K. Solid symbols denote affected members with PD and "m" denotes members with mutation. To provide confidentiality for participants, some unaffected members and some affected members in whom haplotype analysis was not performed are not shown on the trees.

3 different mutations in *LRRK2* exon 41. The G2019S mutation was detected in 8 probands (1 Japanese, 3 Israelis, 1 Moroccan, and 3 Tunisians). Considering the mode of inheritance, 2 (0.5%) of 417 sporadic PD patients had the G2019S with the age at onset of 41 and 42 years, respectively. Including all ethnic groups, 5 ADPD families (2 Israelis and 3 Tunisians) had the G2019S (5 of 130 = 3.8%). Interestingly, 1 (1.3%) of 80 potential or pseudo-ARPD families had the G2019S.

Considering each ethnic group, the frequency of PD probands with the G2019S was as follows: 0.1% (1 of 763) in Asian people, 4.2% (3 of 72) in Caucasian people, and 26.7% (4 of 15) in North African people. The frequency of PD probands with the I2020T was 0.3% (2 of 763) in Asian people. In addition to the pathogenic mutations, we found a novel single polymorphism (6054C>T) that was synonymous (T2018T) in 2 Korean families.

The parent and the parent's sibling of Japanese Patients J1 and J2 with the I2020T were born in the Sagamihara area, and the Japanese Patient K1 with the I2020T was also born near the Sagamihara area. The haplotype with 5 microsatellite markers in this region was exactly the same as that of the Sagamihara family.[11,12] In contrast, the haplotype of the other Japanese Family B (Patients B1 and B2) with the G2019S was quite different from that of the Caucasian families reported previously (Fig. 2).[10]

Clinical Features

Most patients with *LRRK2* mutations in exon 41 showed cardinal signs of idiopathic PD and good responses to levodopa (Table 1). Other clinical features noted in some patients included dementia, hallucination, delusion, and depression. Patients C1 and C2 with the G2019S mutation developed visual hallucinations, paranoidal thoughts, and dementia. Patient H1 with the G2019S mutation developed hallucinations and delusion. Patients B1 and C2 with the G2019S mutation also suffered from depression. Patient J2 with the I2020T mutation developed severe auditory and visual hallucinations and severe delusion. Psychoses were not induced by medication (levodopa/dopamine agonists) and did not relent when *off* medication. The doses of anti-PD drugs for Patient J2 were selegiline 2.5 mg/day, cabergoline 3 mg/day, amantadine 150 mg/day, and talipexole 1.2 mg/

a

Patient		Family (country)																			
		B (Japan)				C (Israel)				D (Israel)				E (Israel)		F (Morocco)		G (Tunisia)		H1 (Tunisia)		I (Tunisia)	
		B1		B2		C1		C2		D1		D2		E1		F1		G1		H1		I1	
Marker	D12S345	210	214	210	232	214	214	214	210	214	218	214	212	214	231	212	224	211	217	214	223	226	234
	D12S2514	295	295	295	295	**291**	291	**291**	291	**291**	295	**291**	295	**291**	291	**291**	291	293	285	**291**	291	291	291
	D12S2515	216	228	216	228	**224**	224	**224**	216	**224**	216	**224**	224	**224**	216	**224**	220	228	228	**224**	212	228	232
(*LRRK2* IVS 30)	D12S2516	252	252	252	252	**254**	254	**254**	252	**254**	252	**254**	252	**254**	254	**254**	254	**254**	254	**254**	254	**254**	254
(*LRRK2* Exon 34)	rs1427263	C	C	C	C	A	A	A	C	A	C	A	C	A	A	A	A	A	C	A	A	A	A
(*LRRK2* Exon 34)	rs11176013	A	A	A	A	G	G	G	A	G	A	G	A	G	G	G	G	G	A	G	G	G	G
(*LRRK2* Exon 34)	rs11564148	T	T	T	T	A	A	A	T	A	T	A	T	A	A	A	T	A	T	A	A	A	A
(*LRRK2* IVS 45)	D12S2518	154	168	154	168	**154**	154	**154**	168	**154**	154	**154**	154	**154**	154	**154**	154	**154**	168	**154**	154	**154**	154
	D12S2519	140	140	140	132	**132**	132	**132**	132	**132**	132	**132**	138	**132**	132	**132**	140	**132**	138	**132**	132	**132**	132
	D12S2520	257	257	257	254	**260**	254	**260**	257	**260**	260	**260**	260	**260**	257	**260**	257	**260**	248	**260**	257	**260**	257
	D12S2521	320	339	320	364	**359**	363	**359**	357	**359**	367	**359**	323	**359**	359	**359**	320	**359**	324	**359**	363	**359**	363
	D12S2522	285	281	285	297	**297**	297	**297**	297	**297**	297	**297**	297	**297**	297	**297**	283	**297**	281	**297**	297	**297**	297
	D12S2523	314	317	314	320	**320**	320	**320**	320	**320**	320	**320**	320	**320**	320	**320**	314	**320**	318	**320**	320	**320**	320
	D12S1653	202	206	202	206	210	218	210	206	210	220	210	220	210	220	210	215	217	206	217	217	217	219

b

Patient		Family (country)						
		J (Japan)				K (Japan)		Sagamihara (Japan)
		J1		J2		K1		
Marker	D12S1631	**185**	181	**185**	177	**185**	177	**185**
	D12S87	**155**	155	**155**	157	**155**	155	**155**
	D12S345	**224**	214	**224**	212	**224**	230	**224**
	D12S1653	**222**	210	**222**	210	**222**	216	**222**
	D12S85	**121**	123	**121**	103	**121**	103	**121**

FIG. 2. Haplotype analysis. **a:** The haplotype of Families C-I with the G2019S from around Europe was similar to the previously reported Caucasian family.[10] Genotypes shared are highlighted in boldface. The haplotype of Japanese Family B with the G2019S was different from that of Families C-I and the previously reported Caucasian family.[10] The size of these markers are corrected using CEPH 1331-01 and -02 as controls. **b:** The haplotype of Families J and K with the I2020T was similar to that of the Japanese Sagamihara family. Shared genotypes are highlighted in boldface. The haplotype with 5 microsatellite markers in this region spans 10.4 cM.

day. Patient B1 developed upward-gaze palsy. None of the patients showed severe autonomic disturbances or amyotrophy. The age at onset of the patients with these three mutations varied widely from 37 to 73 years (average, 55.0 years). In addition to these patients, two grown-up children (aged 36 and 38 years) with mutations were asymptomatic (Family C, Fig. 1b). Pathological studies could not be conducted in all patients with *LRRK2* mutations in this study.

Cardiac MIBG scintigraphy was performed in Patients B1 and B2 with G2019S mutation and Patients J1, J2, and K1 with I2020T mutation (Table 1). For Patient B1, B2, and K1, the H/M ratio was normal. For Patient J1, the H/M ratio was 1.56 in early image and 1.39 in delayed image. For Patient J2, the H/M ratio was 1.45 in early image and 1.30 in delayed image. The cutoff value of delayed H/M ratio is 2.09 in Patients B1 and B2 and 1.45 in Patients J1, J2, and K1 (each cutoff value is different because the MIBG scintigraphy was performed in different hospitals). Patient J2 had no ischemic heart disease, chronic heart failure, diabetes mellitus, or medication history of antidepressants that may influence MIBG uptake. Notably, Patient J2, who developed severe psychosis, had a low H/M ratio in delayed MIBG images.

DISCUSSION

The present study revealed that the *LRRK2* G2019S mutation spreads worldwide across different ethnic groups with variable frequencies. Whereas the G2019S mutation is quite rare in Asia, this mutation is more frequent around Europe[9,13,14] and especially in North Africa. Demographically, it is possible that the distribution and frequencies of this mutation is associated with human migration history. In a previous report, European and North American patients with the G2019S shared a common ancestral haplotype indicative of a single-founder effect.[10] Indeed, our patients from around Europe and North Africa also had the same haplotype. On the other hand, the haplotype of Japanese Family B was quite different from that of Caucasian patients, suggesting they do not share a common founder and they are of independent origin. The possibility that Japanese patients have a common haplotype on the very narrow region could not be excluded. However, it is unlikely that the Japanese G2019S mutation originated from a common and extremely old founder, because the G2019S mutation is rare in Asian people compared with North African and Caucasian peoples.

PARK8 was originally mapped from Japanese Sagamihara family showing late-onset ADPD.[11] The coincidence of haplotype in Japanese Family J, Family K, and Sagamihara family indicates a single-founder effect. The same mutation I2020T was found in a Caucasian patient of European origin (personal communication, Dr. Thomas Gasser),[5] suggesting this mutation also has a worldwide distribution, although the haplotype is unknown at present. Interestingly, G2019S and I2020T mutations affect adjacent codons in the amino acid sequence. In addition to the two mutations, I2012T is also located in the very narrow region within exon 41. This narrow region could be a hot spot and mutations in exon 41 could be relatively frequent. Since *LRRK2* consists of 51 exons, it is important to decide which exon(s) of this gene should be first screened for mutation analysis. In this regard, worldwide screening of exon 41 prior to all other exons is a reasonable strategy. Analysis of other putatively pathogenic coding substitutions and exon 31 is now also recommended.[15]

TABLE 1. *Clinical features of patients (Families A-K) with heterozygous LRRK2* mutations in exon 41

	LRRK2 mutation														
	I2012T	G2019S											I2020T		
Country	Taiwan	Japan		Israel		Israel		Israel	Morocco	Tunisia	Tunisia	Tunisia	Japan		Japan
Mode of inheritance	AD	AR		AD		AD		SP	SP	AD	AD	AD	AD		AD
Consanguinity	−	−		−		−		−	−	−	−	+	−		−
Patient's number	A1	B1	B2	C1	C2	D1	D2	E1	F1	G1	H1	I1	J1	J2	K1
Age at onset (yr)	47	59	73	72	66	64	60	41	42	58	46	37	53	64	43
Disease duration (yr)	14	8	2	13	8	21	18	9	7	2	6	16	8	6	3
Sex	M	F	M	M	F	M	M	F	M	F	F	F	F	F	M
Resting tremor	+	+	+	+	+	+	+	−	+	+	+	+	+	+	−
Rigidity	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Bradykinesia	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Postural instability	−	+	+	+	+	+	+	+	−	−	−	−	−	+	+
Gait disturbance	+	+	+	+	+	+	+	+	+	+	−	−	−	+	+
Clinical response to L-dopa	+	+	+	+	+	+	+	+	+	+	+	+	+	+	NT
Wearing *off*	−	+	+	+	+	+	+	+	+	−	−	−	+	−	−
Asymmetry at onset	−	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Incontinence	−	−	−	−	−	−	−	−	NA	−	−	−	−	−	−
Urinary urgency	−	−	−	+	−	−	−	−	NA	+	−	−	−	−	−
L-dopa–induced dyskinesia	+	−	−	+	+	+	+	+	+	−	−	+	−	−	−
Sleep benefit	−	+	−	−	−	−	−	−	NA	+	−	−	−	NA	+
Dystonia at onset	−	+	−	−	−	−	−	−	−	−	−	−	−	−	−
Hyperreflexia	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−
Psychosis	−	DEP	−	HA, DEL	HA, DEL, DEP	−	−	−	−	−	HA, DEL	−	−	HA, DEL	−
Dementia	−	−	−	+	+	−	−	−	−	−	−	−	−	−	−
Other special comment		Upward gaze palsy													
UPDRS III (*on/off*)	34/57	18/23	29/NA	43/56	38/52	25/33	25/31	16/32	NA	NA	7/37	19/72	7/NA	6/−	15/−
MIBG H/M ratio (early/delay)	ND	2.95/3.08	2.35/2.89	ND	ND	ND	ND	ND	ND	ND	ND	ND	1.56/1.39	1.45/1.30	1.99/1.91

AD, autosomal dominant; AR, autosomal recessive; SP, sporadic; M, male; F, female; MIBG H/M ratio, ^{123}I-metaiodobenzylguanidine heart/mediastinum ratio; ND, not determined; NA, not available; NT, not tried; HA, hallucination; DEP, depression; DEL, delusion; UPDRS, Unified Parkinson's Disease Rating Scale.

With regard to the mode of inheritance, our study showed that not only patients with ADPD but also those with potential ARPD and sporadic PD had *LRRK2* mutations. Moreover, we detected the mutations in late- and early-onset PD. A shared *LRRK2* G2019S haplotype in the vast majority of carriers argues against de novo occurrence.[13] Based on the widely variable age at onset and various modes of inheritance, the phenotype of PD patients with *LRRK2* mutations must be influenced by stochastic, environmental, and other genetic factors.[10]

The clinical features of most patients with *LRRK2* mutations resemble those of patients with typical idiopathic PD.[10] Previously reported patients with *LRRK2* mutations, especially those with G2019S and I2020T, showed no evidence of psychiatric or cognitive dysfunction apart from mild symptoms, despite the long disease duration.[5,11,12,16] However, some of our patients with the *LRRK2* mutations developed severe hallucination, moderate dementia, delusion, and depression, even during the early stages of the disease. Both Patients C1 and C2 with G2019S of the same family had psychosis and dementia. On the other hand, although of the same family, Patient J2 with I2020T showed psychosis and Patient J1 did not show psychosis. Notably, only Patient J2 developed severe psychosis and had low H/M ratio in delayed MIBG images. In contrast, markedly low H/M ratio was not noted in other patients without psychosis.

MIBG scintigraphy is a clinically useful diagnostic method and has high sensitivity. MIBG uptake shows remarkable decrease in Lewy body–positive PD patients from the early stage, except for some of the patients with stage I. Indeed, in a previous study, a more severely reduced H/M ratio was seen in dementia with Lewy bodies compared with PD, and the strong negative correlation between H/M ratio and Hoehn–Yahr stage in PD was observed, suggesting that Lewy body pathology itself may cause low MIBG uptake.[17] Histopathological studies showed a high incidence of Lewy bodies in the sympathetic ganglia and visceral autonomic nervous system[18] and the presence of Lewy bodies and Lewy neurites in the cardiac plexus in PD.[19] These findings may account for low cardiac uptake of MIBG in Lewy body disease, reflecting actual cardiac sympathetic denervation, which precedes the neuronal loss in the sympathetic ganglia.[19,20] In this regard, the reduced cardiac uptake of MIBG in Patient J2 is compatible with Lewy body disease, and Patient J2 with severe psychosis may have cardiac sympathetic denervation and spread of Lewy bodies. On the other hand, Patients B1 and B2 with G2019S and Patients J1 and K1 with I2020T may have no Lewy bodies, similar to most patients of the Sagamihara family.[11,12] The normal MIBG uptake in autosomal recessive juvenile parkinsonism (AR-JP), which is caused by the *parkin* gene,[21,22] generally without Lewy bodies, psychosis, and dementia,[23] supports our findings concerning MIBG scintigraphy and clinical features.

In the present study, the clinical features and cardiac uptake of MIBG varied even in patients with the same I2020T mutation, and also in members of the same family. Indeed, one PD patient with the I2020T mutation from the Sagamihara family had Lewy bodies, although most of the other family members were negative for Lewy bodies (personal communication, Dr. Ogino). Thus, variable pathologies such as positive and negative Lewy body and variable clinical phenotypes were observed in patients with *LRRK2* mutations.

In the previously reported German–Canadian family and western Nebraska family, the phenotypes of *PARK8*-linked parkinsonism with signs of dementia, diffuse Lewy body disease (DLBD), progressive supranuclear palsy (PSP), and motor neuron degeneration showed pathological features of synucleinopathies and tauopathies.[24–27] In the present study, one patient with G2019S showed upward-gaze palsy like PSP, although downward-gaze palsy was not seen. Interestingly, some of the patients with *LRRK2* R1441C mutation had Lewy bodies, while patients with Y1699C mutation in the German–Canadian family and most patients with I2020T of the Sagamihara family had no Lewy bodies.[5,24–27] Thus, clarification of the function of *LRRK2* product and its interaction with other proteins is important for our understanding of the pathogenesis of dopaminergic neuronal death with or without Lewy bodies.

The domain structure of *LRRK2* includes five conserved major functional domains, including leucine-rich repeat (LRR), a Roc (Ras in complex proteins) domain, a COR domain (C-terminal of Roc), a tyrosine kinase catalytic domain (TyrKc), and a WD40 domain.[5] The I2012T, G2019S, and I2020T mutations are located in the kinase domain. The G2019S and I2020T mutations may be involved in the alteration of kinase activities of the *LRRK2* product.[28] Furthermore, this protein may be potentially associated with phosphorylation of both alpha-synuclein and tau.[4,5,29] Indeed, concurrent pathology of alpha-synuclein and tau accumulation within the same aggregates was reported previously.[29] Therefore, the kinase activity of the *LRRK2* product could be a key event in the accumulation and aggregation of these unfolded proteins within the degenerating neurons[4,5] and could be an upstream event.

In our study, we showed that PD caused by *LRRK2* mutations is common and widespread, showing variable pathology and variable clinical phenotypes, including psychosis and dementia. Our study could have major

implications on the clinical understanding of phenotype–genotype correlations and could help in genetic counseling for PD. Insights into the pathogenesis of the disease may lead to new treatment of PD and neurodegenerative disorders.

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Research Review

Recessive Parkinson's Disease

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Abstract: Parkinson's disease (PD) is a progressive neurodegenerative disease caused by loss of dopaminergic neurons in the substantia nigra pars compacta. Although the etiology of PD remains unclear, it is now clear that genetic factors contribute to the pathogenesis of the disease. Recently, several causative genes have been identified in monogenic forms of PD. Accumulating evidence indicates that their gene products play important roles in mitochondrial function, oxidative stress response, and the ubiquitin–proteasome system, which are also implicated in sporadic PD, suggesting that these gene products share a common pathway to nigral degeneration in both familial and sporadic PD. Here, we review recent advances in knowledge about genes associated with recessive PD, including *parkin*, *PINK1*, and *DJ-1*. © 2006 Movement Disorder Society

Key words: *parkin*; *PINK1*; *DJ-1*; recessive Parkinson's disease; mitochondria; oxidative stress; ubiquitin–proteasome system

Parkinson's disease (PD) is the second most common neurodegenerative disease after Alzheimer's disease, affecting ~0.3% of the general population and 3% of people over the age of 65.[1] The discovery of levodopa, a precursor of dopamine, has dramatically improved the functional prognosis of PD. Levodopa, however, is a symptomatic drug and long-term treatment with levodopa is associated with adverse effects, such as motor fluctuations (wearing-off and on–off phenomenon) and dyskinesias. New therapies are therefore required to improve the long-term prognosis. To develop a new remedy for PD, it will be essential to elucidate the pathogenic mechanisms underlying the degeneration of dopaminergic neurons.

Mitochondrial dysfunction and oxidative stress are critical components of most current theories of nigral degeneration in PD.[2–5] However, the mechanisms responsible for the cell death are largely unknown. Recently, there has been increasing evidence that genetic factors play an important role in PD. Although most PD is sporadic, a small proportion of cases shows a Mendelian inheritance. The identification of responsible genes for rare familial forms of PD has provided vital clues to understanding the molecular pathogenesis of the more common sporadic forms of this disease.

To date, at least nine distinct genetic loci have been recognized to be linked to PD (PARK1–3, 5–8, 10, and 11).[6–14] Recently, the family that was originally mapped to PARK4 has been mapped to PARK1.[15] PARK9 has been found not to be a genetic locus for PD because of the atypical associated phenotypes such as spasticity resulting from corticospinal tract degeneration, a supranuclear upward-gaze paresis, and the development of dementia in all affected subjects.[16] Among the PD-associated loci, mutations have been identified in five genes (*α-synuclein*,[17] *parkin*,[18] *PINK1*,[19] *DJ-1*,[20] and *LRRK2*[21,22]) that definitely cause familial forms of PD. *UCH-L1* is another gene that may cause PARK5. So far, only one mutation in the *UCH-L1* gene that is potentially linked to PD was identified in a small German family.[9] Among these genes, mutations in *parkin* (PARK2), *PINK1* (PARK6), and *DJ-1* (PARK7) are associated with

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TABLE 1. *Mutations linked to recessive PD*

	PARK2	PARK6	PARK7
Locus	6q25-27	1p35-36	1p36
Gene	*Parkin*	*PINK1*	*DJ-1*
Inheritance	AR	AR	AR
Number of mutations	> 95	> 17	> 9
Type of mutations	Exonic deletion and duplication, insertion, nonsense, missense	Insertion, deletion, nonsense, missense	Exonic deletion, missense
Lewy body	(−) except for two cases	?	?

autosomal recessive PD, in which loss of function of a single gene product can lead to the degeneration of dopaminergic neurons and the clinical manifestations of parkinsonism (Table 1). This suggests that these gene products are essential for the survival of nigral neurons. Therefore, identification of the physiological functions of these proteins is expected to lead to elucidation of the pathogenic mechanisms underlying the disease. Here, we review the clinical findings in recessive forms of PD and the molecular biology of *parkin*, *PINK1*, and *DJ-1*.

PARKIN (PARK2)

Clinical manifestations of *parkin*-associated PD were originally characterized by parkinsonism associated with early onset before the age of 40, wearing-off phenomenon, and slow disease progression.[23] More recent studies have suggested a broader phenotypic spectrum of *parkin*-associated PD that includes later age at onset and tremor-dominant manifestations without foot dystonia, hyperreflexia, diurnal fluctuations, sleep benefit, and early susceptibility to levodopa-induced dyskinesias.[24–26] Many *parkin*-associated PD patients, therefore, appear to be clinically indistinguishable from sporadic PD. Remarkably, a wide variation of age at onset has been reported, even within single families with mutations in the *parkin* gene,[26] suggesting that additional factors, either genetic or environmental, contribute to the phenotype. However, Lohmann and colleagues[27] have reported that *parkin*-associated PD patients tend to have earlier and more symmetrical onset, slow progression of the disease, and greater response to levodopa despite low doses compared with those with early-onset PD without *parkin* mutations. Clinical features including atypical phenotypes are summarized in Table 2.[27–31] At present, there appears to be no genotype–phenotype correlation. Histopathological characteristics of *parkin*-associated PD are neuronal loss and gliosis limited to the substantia nigra and locus coeruleus. Although the entire knowledge on the pathology is based on limited cases, Lewy bodies, which are cytoplasmic proteinaceous inclusion bodies that are considered the hallmark of PD, are absent[28,29,32–37] except in two reported cases with compound heterozygous mutations.[38,39] Atypical pathological findings have been reported in *parkin*-associated PD, such as tau pathology in the cerebral cortex and brainstem nuclei,[29,32] degeneration of the spinocerebellar system,[28] and α-synuclein–positive inclusions in the neuropils of the pedunclopontine nucleus.[36] Presence of α-synuclein–positive inclusions including Lewy bodies, although detected in only a small number of patients, suggests a possible relationship between *parkin*-associated PD and idiopathic PD.

The gene responsible for *parkin*-associated PD, *parkin*,[18] contains 12 exons spanning over 1.4 megabases and encodes a 465 amino acid (aa) protein with moderate homology to ubiquitin at the amino terminus (ubiquitin-like domain, Ubl) and two RING finger motifs at the carboxy terminus. Various *parkin* mutations have been reported worldwide, including exonic deletions, exonic duplications, insertions, and many different point mutations.[26,30,31,40] Mutations in the *parkin* gene are a relatively frequent cause of early-onset PD, especially in cases with a positive family history and an autosomal recessive mode of transmission. To date, more than 95 different mutations in approximately 400 patients have been reported worldwide.[41] Frequency of the mutations in early-onset PD has been estimated at 40% to 50% in

TABLE 2. *Clinical features of* parkin*-associated PD*

Age of onset usually < 40 years
Typical presenting phenotype: young-onset PD
Normal cognition
Frequent foot dystonia
Early instability, freezing, festination, or retropulsion in some cases
Dramatic response to levodopa, and dose-sensitive motor and psychiatric complications of medication
Excellent response to anticholinergics in some cases
Usually benign and slow disease course
Atypical presenting phenotypes include:
Later onset, mimicking idiopathic PD
Psychosis, panic attacks, depression, hypersexuality, obsessive–compulsive behavior
Exercise-induced dystonia
Atremulous bilateral akinetic rigid syndrome
Focal dystonia (writer's cramp, cervical)
Autonomic or peripheral neuropathy
Cerebellar and pyramidal tract dysfunction

familial cases[26,42] and at 10% to 20% in idiopathic cases.[26,30,31,42] It is noted that a considerable number of patients carry a single heterozygous mutations. Clinical features of the carriers of heterozygous *parkin* mutations are more similar to those of idiopathic PD, including a significantly later age at onset and more asymmetric disease presentation, than those of carriers of two mutations.[27,42–44] Since *parkin*-associated PD is considered to be an autosomal recessively transmitted disease, the role of mutations in single heterozygous state is difficult to interpret. There are two possibilities: one is haploinsufficiency, the other dominant negative effect. In addition, a possible explanation is that still unknown factors such as mutations located elsewhere in the genome[45] and environmental factors act in combination to cause the disease. On the other hand, in a North American study of more than 300 PD patients unselected for age at onset or family history, the frequency of *parkin* mutations reported as pathogenic in homozygous or compound heterozygous individuals is essentially the same in PD patients (3.8%) and controls (3.1%).[46] Thus, whether single heterozygous mutations in *parkin* are associated with PD remains to be elucidated.

The gene product, parkin, is localized to the Golgi complex in addition to the cytoplasm in human brain, although parkin has no transmembrane domain.[47] In cultured cells and rat brain, parkin also associates with synaptic vesicles as a peripheral membrane protein.[48] Recently, parkin was implicated in the ubiquitin–proteasome system (UPS) as an E3 ubiquitin ligase, and mutations in the *parkin* gene is reported to result in loss of ligase function.[36] The UPS is involved in two tasks. One is the accurate timely regulation of the level of short-lived proteins that plays a role in processes such as cell cycle regulation, signal transduction, and metabolism. The other task is protein quality control. Polyubiquitination of the target proteins for degradation by proteasomes is catalyzed by three enzymes, E1 (ubiquitin-activating enzyme), E2 (ubiquitin-conjugating enzyme), and E3 ubiquitin ligase. Parkin has been shown to catalyze the proteasomal degradation of target proteins by interacting with E2 and target proteins through its RING domain[49–51] and by binding the Rpn10 subunit of 26S proteasomes through its Ubl domain.[52] Parkin is also reported to exhibit E3 activity by interacting with components of SCF complexes (Skp1–Cullin–F-box protein).[53] In addition, parkin forms a complex with Hsp70 and CHIP, resulting in enhancement of its E3 enzymatic activity.[54] Since *parkin*-associated PD is recessively inherited, that is, loss of function of parkin leads to development of *parkin*-associated PD, substrates for parkin (for its E3 function) would be expected to accumulate in the brain. Substrates identified to date include cell division control–related protein 1 (CDCrel-1),[50] parkin-associated endothelin receptor–like receptor (Pael-R),[51] the *O*-glycosylated form of α-synuclein (αSp22),[55] synphilin-1,[56] synaptotagmin XI,[57] SEPT5_v/CDCrel-2,[58] cyclin E,[53] the p38 subunit of the aminoacyl–tRNA synthetase complex,[59] and α/β-tubulin.[60] In addition to the substrates, several interactive molecules have been reported.[61] One may postulate that the substrates that escape from ubiquitination by parkin for degradation by the proteasome accumulate in *parkin*-associated PD brains, leading to nigral degeneration. In fact, three of the substrates (CDCrel-1,[58] Pael-R,[51] αSp22[55]) have been reported to accumulate in *parkin*-associated PD brains. However, an important caveat is that most substrates have been identified by in vitro experiments and remain to be validated.[62] On the other hand, parkin has recently been shown to catalyze lysine63 (K63)-linked ubiquitination, which is not recognized by the proteasome.[63,64] Assembly of polyubiquitination occurs through the sequential formation of an isopeptide bond between the carboxy-terminal glycine residue and specific lysine residues in ubiquitin. Substrates tagged with the polyubiquitin chain linked via K29 or K48 are recognized and degraded by the proteasome.[65] In contrast, K63-linked ubiquitination is involved in diverse cellular processes such as endocytosis[66–69] and protein sorting and trafficking.[70–72] Although the relevance of K63-linked ubiquitination by parkin in the pathogenesis of *parkin*-associated PD remains unclear, this insight might facilitate elucidation of the pathogenesis.

With regard to loss of function of parkin as a mechanism underlying *parkin*-associated PD, *parkin*-knockout animal models should help to elucidate the mechanisms of the disease (Table 3). Although three lines of *parkin*-knockout mice have been reported,[73–75] none of these develops a PD-like phenotype or PD pathology. Two exon 3–disrupted knockouts show subtle motor and cognitive deficits, inhibition of amphetamine-induced dopamine release and glutamate neurotransmission, and abnormal dopamine metabolism, but no loss of substantia nigra or locus coeruleus catecholaminergic neurons. Meanwhile, an exon 7–disrupted knockout exhibits loss of noradrenergic locus coeruleus neurons. Interestingly, none of the previously reported substrates for parkin in the UPS accumulated in the knockout brains,[74,75] suggesting that parkin might function as an E3 for K63-linked ubiquitination. Proteomic analysis of ventral midbrain from the exon 3–disrupted mice revealed decreased abundance of a number of proteins involved in mitochondrial function or oxidative stress.[76] Consistent with reduction of these proteins, the mice exhibited de-

TABLE 3. *Animal models of* parkin *and* DJ-1 *PD*

Knockout models	Phenotypes	Pathology
Parkin		
Exon 3-knockout mice[73]	Subtle motor and cognitive deficits, inhibition of amphetamine-induced dopamine release, inhibition of glutamate neurotransmission, increased dopamine level	Normal brain morphology
Exon 3-knockout mice[74]	Subtle behavioral deficits, increased extracellular dopamine concentration, reduced synaptic excitability	Normal brain morphology
Exon 3-knockout mice[75]	Reduced norepinephrine-dependent startle response	Loss of catecholaminergic neurons in the locus coeruleus
Drosophila null mutants[77]	Reduced life span, locomotor defects, male sterility	Muscle fiber degeneration, mitochondrial pathology
Drosophila null mutants[78]	Low mass, reduced life span, locomotor defects, sterility	Muscle fiber degeneration, mitochondrial defects
DJ-1		
Exon 2-knockout mice[122]	Hypoactivity in the open field, reduced evoked dopamine overflow due to increased reuptake, less sensitivity to the inhibitory effects of D2 autoreceptor stimulation	Normal brain morphology
Promoter-exons 1-5—knockout mice[123]	Age-dependent and task-dependent motoric behavioral deficits, increased dopamine reuptake rates, elevated tissue dopamine content	Normal brain morphology
Exons 3-5 with modified first coding exon[124]	Depression in amphetamine-induced locomotor activity, increased striatal denervation and dopaminergic neuron loss induced by MPTP	Normal brain morphology

creased respiratory capacity of striatal mitochondria, reduced serum antioxidant capacity, and increased protein and lipid peroxidation. A recent study in Drosophila with an inactivated orthologue of human *parkin* also suggested mitochondrial dysfunction.[77] The *parkin*-null mutants exhibited reduced longevity, locomotor defects, and sterility.[77,78] Mitochondrial pathology has been the earliest manifestation of muscular degeneration and defective spermatogenesis in the *parkin* mutants. These observations suggest a new role for parkin in the regulation of normal mitochondrial function. Parkin overexpression in cultured cells protects against mitochondria-dependent apoptosis and parkin associates with the outer mitochondrial membrane.[79] Furthermore, parkin has been shown to have cytoprotective effects against diverse cellular insults, including dopamine-mediated toxicity,[80] kainate-induced excitotoxicity,[53] overexpression of Pael-R[51] or the p38 subunit,[59] and toxicity induced by proteasomal inhibition or overexpression of mutant α-synuclein.[81] Recently, antisense knockdown of *parkin* was reported to cause apoptotic cell death of human dopaminergic cells associated with caspase activation, accompanied by accumulation of oxidative dopamine metabolites due to auto-oxidation of DOPA and dopamine.[82] These results suggest that parkin may function as a multipurpose neuroprotectant.[83] Interestingly, parkin has been shown recently to be *S*-nitrosylated in vitro, as well as in brains of patients with idiopathic PD.[84,85] Chung and colleagues[84] reported that *S*-nitrosylation of parkin impaired its E3 activity and its protective function. On the other hand, Yao and colleagues[85] showed that *S*-nitrosylation increased, rather than decreased, the E3 activity of parkin. Several technical differences may explain the discrepancy between them.[86] These findings may thus provide a molecular link between *parkin*-associated PD and sporadic PD.

PINK1 (PARK6)

Although a small number of studies have been published, the clinical manifestations of *PINK1*-associated PD are characterized by parkinsonism associated with good response to levodopa, frequent occurrence of levodopa-induced dyskinesias, and infrequent occurrence of dystonia, very similar to idiopathic PD. The only distinctive features are an earlier age of onset (18 to 51 years) and slower disease progression.[10,87–95] Hyperreflexia and sleep benefit are not common. Some patients, however, exhibit foot dystonia at onset and sleep benefit, thereby resembling *parkin*-associated PD.[88,89,2,94] Psychiatric symptoms such as hallucination, depression, and anxiety are reported in some patients.[88,89,92,94] Dementia is described in one Israeli patient.[88,89] Autopsy data are not available.

The gene responsible for *PINK1*-associated PD, *PINK1* (PTEN-induced kinase 1), was previously cloned by two groups examining gene regulation by the tumor suppressor PTEN[96] and differential gene expression in mouse cell lines that vary in their metastatic potential.[97] The frequency of *PINK1* mutations (homozygous or compound heterozygous) has been estimated at 3.3% among Italian sporadic early-onset PD.[93,94] Hatano and colleagues[88,89] have reported that 6 (~15%) of 39 early-onset autosomal recessive families, mostly Asian, had homozygous or compound heterozygous *PINK1* muta-

tions. Interestingly, despite autosomal recessive transmission, 5% of the sporadic early-onset PD have single heterozygous *PINK1* mutations.[93,94] Although only small number of reports are available so far,[87,89–91,93] the clinical features between the homozygous and heterozygous patients are generally similar, but onset age is about 10 years earlier in the former than in the latter. The role of single heterozygous *PINK1* mutations in PD remains to be clarified. *PINK1* contains eight exons spanning 18 kilobases (kb) and encodes a 581 aa protein with a 34 aa mitochondria targeting signal and a highly conserved protein kinase domain (residues 156 to 509) similar to serine/threonine kinases of the Ca^{2+}/calmodulin family.[19] Most mutations have been reported to cluster in or around the putative kinase domain,[19,89–95] suggesting that the loss of PINK1 kinase activity may cause the disease. The kinase substrates for PINK1 have not yet been identified. In cultured cells, transfected PINK1 is localized in the mitochondria and exerts a protective effect against stress induced by proteasome inhibitors, which is abrogated by the mutations,[19] although how PINK1 protects the cells and whether PINK1 kinase activity plays a role in protection is not clear. Interestingly, PINK1, particularly the N-terminally processed mature form, has been shown to exist in the cytosol as well as in mitochondria.[98] Recently, two mutations found in patients, G309D and L347P, were associated with reduced kinase activity.[98] The L347P mutation markedly decreases the stability of PINK1 and consequently reduces its kinase activity, whereas G309D has much more modest effects on these parameters in vitro. The relevance of PINK1 kinase activity for the pathogenesis of *PINK1*-associated PD thus awaits further clarification.

DJ-1 (PARK7)

The clinical features of *DJ-1*–associated PD, which cannot be fully appreciated as yet because only a few studies have been published, are similar to *parkin*-associated PD and *PINK1*-associated PD, namely, parkinsonism associated with an early age of onset (17 to 42 years), good response to levodopa, slow progression, and levodopa-induced dyskinesias.[11,99–103] Behavioral and psychic disturbances at onset or early stages in the disease course and focal dystonias, including blepharospasm, have been reported.[11,99] Brain single positron emission computed tomography with dopamine transporter tracer showed severe abnormalities consistent with presynaptic dysfunction of nigrostriatal dopaminergic systems.[11] The gene responsible for *DJ-1*–associated PD, *DJ-1*, contains eight exons spanning over 24 kb and encodes a 189 aa protein that belongs to the ThiJ/PfpI family.[20] A number of pathogenic mutations have been identified in the *DJ-1* gene, including exonic deletions, truncations, and homozygous and heterozygous missense mutations.[20,100–105] However, the frequency of the mutations appears to be low.[95,102] It has been reported that only 2% of early-onset PD have *DJ-1* mutations.[102] DJ-1 is ubiquitously expressed, including in brain, where both neurons and glia are immunopositive for the protein.[106,107] Interestingly, DJ-1 is not an essential component of Lewy bodies, but localized in glial cytoplasmic inclusions in multiple-system atrophy, and in neuronal tau inclusions of tauopathies such as Pick's disease, corticobasal degeneration, progressive supranuclear palsy, and Alzheimer's disease.[106,108,109] In addition, DJ-1 levels are markedly increased in the detergent-insoluble fraction of brains of patients with sporadic PD.[110] These findings suggest that DJ-1 might have diverse functions. Indeed, DJ-1 appears to be implicated in multiple processes, including chaperone activity,[111] protease activity,[107] and the oxidative stress response.[112–115] The crystal structure of human DJ-1 has been resolved and shows that DJ-1 exists in solution as a homodimer, and the mutation found in patients disrupts the dimerization.[116–119] Although the biological function of DJ-1 remains elusive, there is growing evidence that DJ-1 functions as an antioxidant protein and/or as a redox sensor of oxidative stress. DJ-1 has been shown to demonstrate an acidic shift in isoelectric point in cultured cells under oxidative stress owing mainly to oxidative modification of cysteine residues within the molecule.[112–115] DJ-1 eliminates hydrogen peroxide in vitro by oxidizing itself.[113] Moreover, overexpression of DJ-1 protects against oxidative injury, whereas knockdown of *DJ-1* by short interfering RNA enhances the susceptibility to oxidative stress.[113,120] DJ-1 also protects against oxidative stress through its cysteine–sulfinic acid–driven mitochondrial localization.[121] Interestingly, DJ-1 interacts with parkin under conditions of oxidative stress,[110] suggesting that these proteins may cooperate in a common neuroprotective pathway. Indeed, DJ-1 exhibits a protective effect against the toxicity induced by overexpression of Pael-R, a substrate for parkin, in addition to endoplasmic reticulum stress and proteasomal inhibition.[120] These results also suggest that DJ-1 functions as a molecular chaperone or protease for refolding or degradation of misfolded or aggregated proteins induced by endoplasmic reticulum stress and proteasomal inhibition. Very recently, three reports describe *DJ-1* knockout mice generated by targeted disruption of exon 2,[122] the first five exons and part of the promoter,[123] or exons 3–5 with modified first coding exon.[124] Although none of these models demonstrate alteration in gross brain morphology or dopamine neuron loss, two lines of

knockouts[122,123] exhibit abnormalities in striatal dopaminergic function with motor deficits. Interestingly, these two lines of mice show increased dopamine reuptake, suggesting that DJ-1 plays an essential role in normal dopaminergic physiology. The other knockout[124] shows increased striatal denervation and dopaminergic neuron loss induced by 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP), suggesting protective effect of DJ-1 against dopaminergic insults.

CONCLUSIONS

Based on clinical features, differentiating these three forms of recessively inherited PD, sometimes even from idiopathic PD, appears to be difficult, as similar neuronal groups, notably dopaminergic neurons, are predominantly affected. Genetic analysis is therefore essential for an accurate diagnosis. Genetic testing has become commercially available for these genes (MRC-Holland, Amsterdam, The Netherlands). The kit seems to have the advantage of a relatively low running costs, dealing with large numbers of samples, and low investment for setting. A point to note is that the kit is not suitable to detect new mutations. The clinical and pathological similarities suggest that common molecular pathways may contribute to the pathogenic mechanisms of both recessive and sporadic PD. Mitochondrial dysfunction and oxidative stress are thought to play a prominent role in the pathogenesis of sporadic PD.[2–5] Furthermore, impairment of the UPS with protein mishandling has recently been suggested to be a major pathway leading to neurodegeneration in sporadic PD.[124,125] Although the mechanisms of these abnormal events in the neurons of sporadic PD are still unknown, recent advances in recessive PD have implicated parkin, PINK1, and DJ-1 in mitochondrial function, oxidative stress, and the UPS, as described above. This growing evidence suggests that loss of function of parkin, PINK1, and/or DJ-1 might contribute to the pathogenesis of sporadic PD. Further studies of recessive PD and associated gene products will help elucidate the molecular relationships between recessive and sporadic PD and hopefully lead to development of new strategies for nigral protection.

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Nuclear localization of the 20S proteasome subunit in Parkinson's disease

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Abstract

Considering the involvement of ubiquitin–proteasome system (UPS) in Parkinson's disease (PD), the aim of the present study was to determine the distribution of proteasomes in PD brains. Immunohistochemical studies showed localization of 20S proteasome in the nuclei of neurons of the putamen and substantia nigra of PD. In contrast, no nuclear staining was observed in the same areas of brains of controls. Our results suggest that nuclear localization of 20S proteasome seems to be associated with the pathogenesis of PD.

Keywords: Parkinson's disease; 20S proteasome; Immunohistochemistry

Parkinson's disease (PD) is a neurodegenerative disorder characterized clinically by tremor, rigidity, bradykinesia and postural instability. Pathologically, PD is characterized by selective loss of neurons in the substantia nigra (SN) pars compacta, and the presence of eosinophilic inclusions, known as Lewy bodies (LBs) [12]. How and why LBs are formed in PD patients is not yet clear. However, LB components have been well investigated.

Our understanding of the PD disease process has expanded tremendously over the past few years through the discovery of gene products for monogenic familial PD although most of the patients with PD are idiopathic. Especially, the discovery of α-synuclein provided excellent information on the mechanisms of LB formation [12]. In addition, parkin and UCH-L1 are directly linked to the ubiquitin–proteasome pathway (UPP) as a component of this system [6]. Thus, the UPP seems to play an important role in the pathogenesis of PD.

With regard to the mechanisms of LB formation, identification of several components of the inclusion bodies provides valuable information. In this context, ubiquitin itself is a key protein that has been considered as a suitable marker for LBs [22]. Apart from ubiquitin, gene products, such as α-synuclein and UCH-L1 have been reported to be components of LB [7,21]. Furthermore, additional component, such as symphillin-1 has been identified also [24]. Therefore, there is growing evidence that UPP could be involved not only in the pathogenesis of the disease but also LB formation. Indeed, previous studies demonstrated that proteasome inhibitors increase the frequency of ubiquitin-positive intracellular inclusions like LBs [23].

The 20S proteasome, a multicatalytic proteinase, combines with 19S regulator complex to form the 26S proteasome, which is responsible for ubiquitin–ATP-proteolysis [14,20]. Numerous studies have recently emphasized the biological importance of the UPP, which is capable of catalyzing rapidly, timely, and unidirectionally a multitude of biological reactions including cell-cycle progression, DNA repair, cell death, signal transduction, transcription, metabolism, and immunity [5,16,25]. Several studies of proteasome activities in the SN of PD have been reported so far, though it is controversial whether the activities of the 20S proteasome decrease in PD. Proteasomes are present in the nucleus and in the cytoplasm of various types of cells [18]. In fact, immunoelectron microscopic studies of the rat central nervous system revealed that proteasomes are present not only in the cytoplasm but also in the nucleus in neurons, dendritic and axonic processes [11].

The aim of the present study was to characterize the distribution of 20S proteasome in PD patients. For this purpose, we analyzed immunohistochemically the postmortem brains of patients with idiopathic PD using antibody of proteasome subunit.

We performed immunohistochemical studies using samples from the putamen obtained from four patients with PD and four control subjects and from the SN obtained from three patients with PD and two control subjects. The clinical profiles of the patients are summarized in Table 1. We obtained informed consents from the families of all patients and control subjects.

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Table 1
Clinical summary of participating subjects

Case	Diagnosis	Age (years)	Sex	Duration (years)	Postmortem delay (h)	Cause of death	Sample
1	PD	51	M	11	3.5	Pneumonia	P1, N1
2	PD	77	M	7	6.5	Pneumonia	P2, N2
3	PD	65	F	4.5	2	Asthma	P3
4	PD	85	F	6	2	Pneumonia	P4
5	PD	72	F	5	<24	Lung hemorrhage	N3
6	PD	69	M	22	9	Malignant syndrome	P (Fr)
7	Control	62	M	–	11	AML	P1, N1
8	Control	66	F	–	1	Ovarian cancer	P2
9	Control	38	M	–	10	Malignant lymphoma	P3
10	Control	41	M	–	2.5	CRF	P4
11	Control	65	F	–	1.5	Pachymeningitis	N2
12	Control	99	M	–	<24	Pneumonia	Pons
13	Control	47	F	–	2	Gastric cancer	P (Fr)

AML: acute myelocytic leukemia, CRF: chronic renal failure, F: female, Fr: fresh frozen brain, M: male, N: substantia nigra, P: putamen, PD: Parkinson's disease.

The pathological diagnosis was confirmed in all patients and age-matched control subjects in the Department of Neurology, Juntendo University School of Medicine. The study protocol was approved by the Human Ethics Review Committee of Juntendo University School of Medicine.

A mouse monoclonal antibody to 20S proteasome subunit α6 (PW8100) was purchased from Affinity Research Products (Mamhead Castle, UK). The specificity of this antibody has been confirmed previously [4]. Schmidt et al [19] examined immunohistochemistry using this antibody to pontine samples obtained from control subjects and SCA3 patients. They showed strong staining in the majority of nuclei of pontine neurons in control subjects. We obtained the same results using antibody to α6 (PW8100) subunit of the 20S proteasome. We also used another mouse monoclonal antibody to the 20S proteasome subunit α6 for SN samples, which was kindly provided by Professor Keiji Tanaka, Department of Molecular Oncology, The Tokyo Metropolitan Institute of Medical Science. This antibody was used for Western blot using putamen sample of Case No. 6. The putamen sample was placed into ice-cold homogenization buffer (0.32 M sucrose and 4 mM 4-(2-hydeoxyethl)-1-piperazineethanesulgonic acid (HEPES)–NaOH, pH 7.4) and homogenized using a Potter–Elvehjem homogenizer in the presence of a mixture of protease inhibitors (Complete Mini EDTA-free, Roche Diagnostics, Penzberg, Germany). After centrifugation at 600 × *g*, the supernatant was used for analysis. Sample was separated by 10% sodium dodecyl sulfate–polyacrylamide gel electrophoresis. Transferred protein was probed with the anti α6 antibody and visualized using a chemiluminescence reagent.

Immunohistochemical experiments were conducted as described previously [2]. Formalin-fixed paraffin-embedded sections (6 μm thick) were deparaffinized with xylene and hydrated in ethanol. The deparaffinized sections were microwaved in phosphate buffer (Antigen Retrival Citra, BioGenex, San Roman, CA) at high power for 3 min and low power for 5 min for antigen retrieval. Endogenous peroxidases were quenched by incubation with 3% hydrogen peroxide for 5 min. After blocking the slides with 10% normal goat serum in phosphate buffered saline (PBS) for 20 min at room temperature, the sections were treated overnight at 4 °C with primary antibody. After the treatment, the sections were incubated with biotinylated anti-mouse IgG from goat (Dako Corporation, Carpinteria, CA; diluted 1:200) as the second antibody. After incubation with streptavidin conjugated to horseradish peroxidase (HRP) (Dako; 1:200), we treated the sections with biotinyl tyramide and hydrogen peroxide. Sections were then incubated with streptavidin conjugated to HRP and visualized by 3′,3′-diaminobenidine (DAB).

Double immunofluorescence was performed using putamen sections of 2 PD brain (Case No. 1, 2) with anti-α6 subunit of proteasome (PW8100) and anti-Oct-1 (Santa Cruz Biotechnology, Santa Cruz, CA) antibodies. The anti-Oct-1 antibody was used as a marker for cell nuclei. These sections were treated with Alexa fluor goat anti-rabbit IgG (Molecular Probe, Eugene, OR; diluted 1:300) and Alexa fluor goat anti-mouse IgG (Molecular Probes, Eugene, OR; diluted 1:300). The signal was examined under Zeiss LSM 510 laser scanning confocal microscope (Zeiss, Oberkochen, Germany).

For semiquantitative analysis, microscopic photographs of the whole SN and lesions of putamen nuclei were prepared. Two independent observers blinded to the clinical information counted the number of anti-α6-positive neurons in the SN and putamen. Differences between groups were examined for statistical significance using one-way ANOVA. A P value less than 0.05 was considered significant.

Fresh frozen putamen-containing brain sections of PD (Case No. 6) and control subject (Case No. 13) were obtained for Western blot analysis. The samples were placed into ice-cold homogenization buffer (0.32 M sucrose and 4 mM HEPES–NaOH, pH 7.4) and homogenized using a Potter–Elvehjem homogenizer in the presence of a mixture of protease inhibitors (Complete Mini EDTA-free, Roche Diagnostics). After centrifugation at 600 × *g*, the pellet was collected and used for analysis.

Samples were 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 a 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

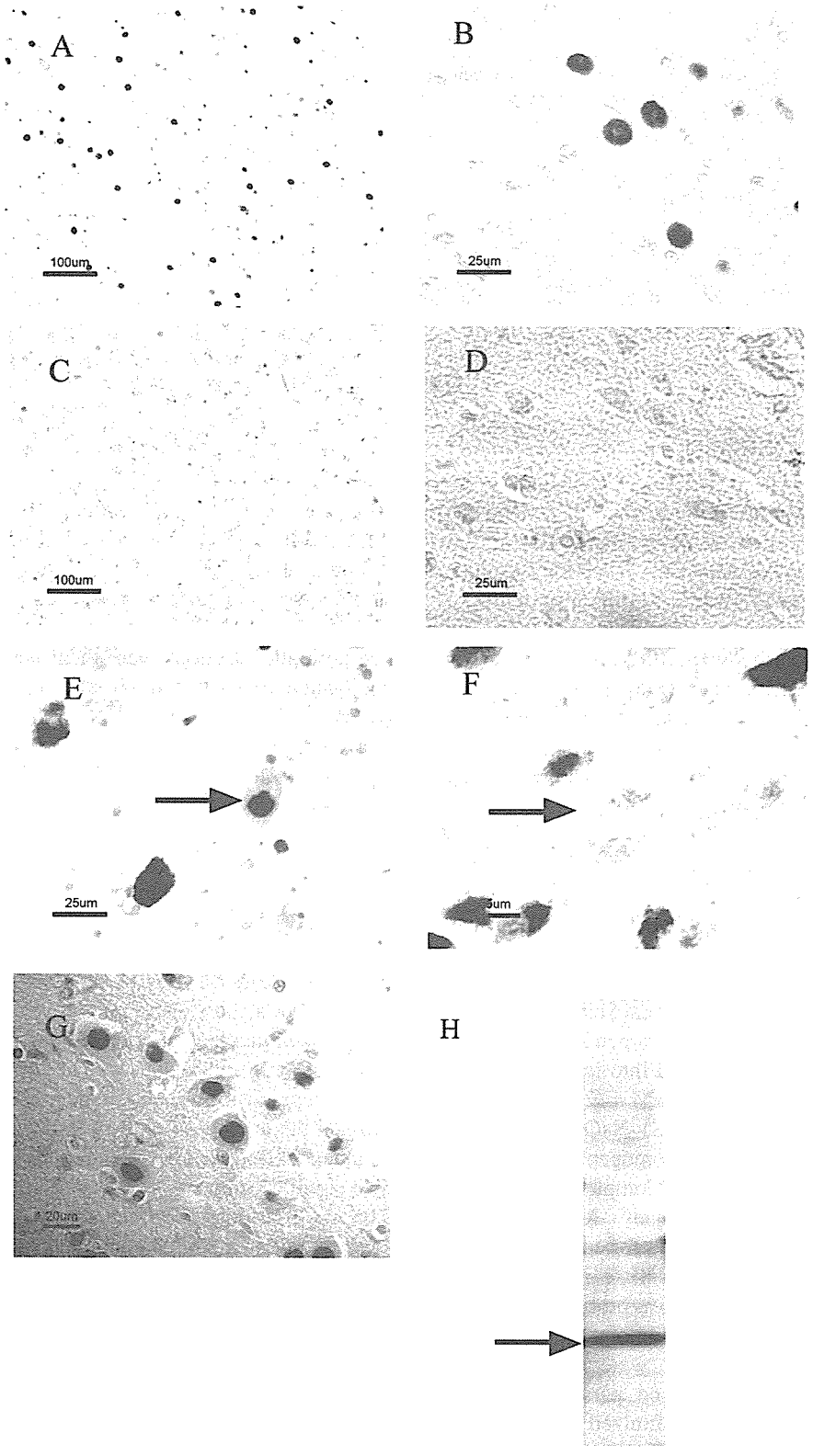

Fig. 1. Immunohistochemical staining of the striatum in a Parkinson's disease (PD) brain (A, B) and control brain (C, D) using antibodies to the α6 subunit of the 20S proteasome (PW8100) (A–D). Note the prominent nuclear staining for 20S proteasome in PD and the very weak or negative staining in the control. In addition, nuclear staining of 20S proteasome was observed in substantia nigra of PD (E) and control (F) in sections stained with antibody to the α6 subunit of the 20S proteasome (provided kindly by Professor Keiji Tanaka). Arrow indicates the body of a nigral neuron. In contrast, no staining for this subunit was observed in the control (F). Arrow indicates the body of a nigral neuron. The dark-brownish color products represent neuromelanines. (G) Staining using antibody to the α6 subunit of the 20S proteasome (PW8100) in pontine section of a control subject. (H) A single band of about 30 kDa, which is equivalent to the expected molecular weight of α6 subunit (from Professor Tanaka), was detected in PD.

antibodies at 4 °C. The primary antibodies were α6 proteasome subunit (PW8100) and β-tubulin as an internal marker (Santa Cruz Biotechnology). After incubation with HRP-conjugated secondary antibodies, the reaction was visualized using a chemiluminescence reagent.

In putamen sections of all PD brains, most nuclei of neurons showed strong staining by α6 subunit of the 20S proteasome antibody (Fig. 1A and B). In contrast, proteasome immunoreactivity was rarely observed in the nuclei of putamen neurons of the control brains (Fig. 1C and D).

Similar results were obtained when using the α6 subunit of the 20S proteasome antibody in the SN in PD and controls (Fig. 1E and F). Most nuclei of neurons showed strong staining in all three PD samples of SN. No immunoreactivity was noted in other regions of SN, locus coeruleus, dorsal vagal nucleus, or nucleus basalis of Mynert. Similar results (see Schmidt et al. [19]) were obtained when using the α6 subunit of the 20S proteasome antibody (PW8100) in pontine sections of control subjects (Fig. 1G). Anti-α6 subunit of the 20S proteasome antibody demonstrated a single band of about 30 kDa corresponding to the α6 subunit of 20S proteasome in the PD brain (Fig. 1H).

To confirm the localization of α6 subunit of the 20S proteasome in the nuclei of neurons, we performed double immunofluorescence staining with anti-α6 subunit of the 20S proteasome and Oct-1 antibodies using putamen of two PD patients. The results confirmed the localization of α6 subunit in the nuclei in PD brain (Fig. 2A–C). Semiquantitative analysis showed that the proportions of α6-positive neurons to all neurons of the SN and putamen were higher in PD that in control subjects. Repeated paired analyses with Boniferroni's correction showed significant differences between PD and control subjects ($P < 0.05$, Fig. 3A and B). Furthermore, Western blot analysis using insoluble fractions of the brain demonstrated a higher protein level of α6 subunit in PD patient compared with the control (Fig. 3C).

In the present study, we demonstrated the distribution of proteasomal subunits in idiopathic PD by immunohistochemistry. In PD brains, most nuclei of putamen neurons showed strong staining when we used antibodies against the subunits of 20S proteasome. A similar immunohistochemical distribution was observed in the SN, although only few neurons were noted in this area of PD patients. We also performed immunohistochemical studies using other subunits of 20S proteasome, such as α2, 3, 4, 5, 7, β1, 3, 5 in four patients with PD (age: 69 ± 8.6 years, disease duration: 11.03 ± 4.9 years, postmortem delay 6.5 ± 5.5 h, mean ± S.D.) and four control subjects (age: 62 ± 14.8 years, postmortem delay 4.0 ± 3.0 h, mean ± S.D.). Immunostaining for all the subunits was also observed in the nuclei, similar to the pattern seen for the α6 subunit (data not shown). In contrast,

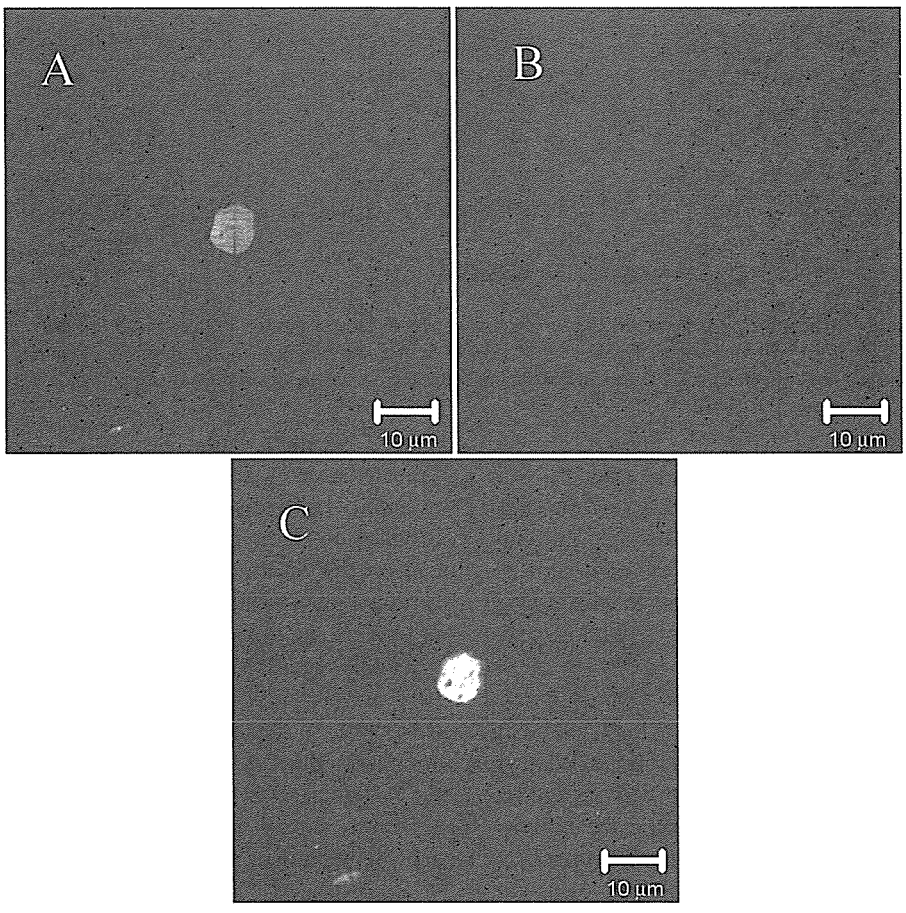

Fig. 2. Localization of α6 subunit in the nuclei of PD neurons. Double immunofluorescence staining of putamen neurons of PD with antibody for α6 subunit of the 20S proteasome, PW8100 (green in A) and Oct-1 (red in B) together with the merged image (C). Note the colocalization of α6 subunit of the 20S proteasome (green in A) and Oct-1 (red in B) in PD neurons.

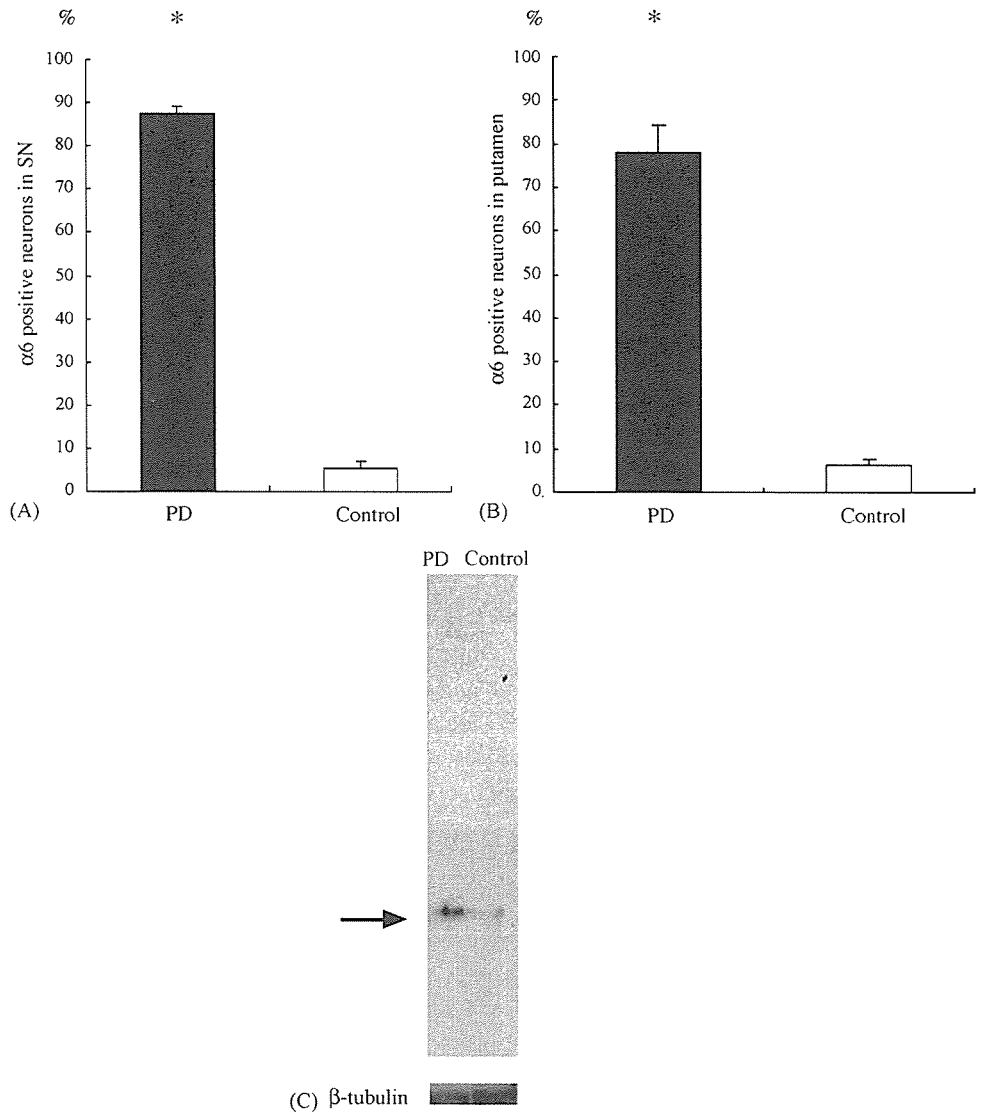

Fig. 3. Semiquantitative analysis of α6 proteasome and protein. The percentages of α6 proteasome-positive neurons in SN (A) and putamen (B) were significantly higher in SN and putamen of PD than the control (data are mean ± S.E.M., $P < 0.05$, one way ANOVA and Bonferroni's correction). (C) Immunoblotting of α6 proteasome (PW8100) in soluble fractions of putamen of PD patient and control subject. Note the higher α6 proteasome protein level in PD than control. β-tubulin is used as internal marker.

immunostaining of the frontal cortices of three patients with PD (age: 69 ± 15 years, disease duration: 10 ± 3.9 years, postmortem delay 3.0 ± 0.5 h, mean ± S.D.) using the same antibody (PW8100), was negative for the α6 subunit (data not shown). These findings suggest that the specific nuclear localization of α6 in nigrostriatal pathway may play a role in the pathogenesis of PD or the disease process.

Several studies demonstrated altered distribution of proteasome subunits during different phases of the cell cycle [1,15], in stress conditions (e.g., glucose starvation and hypoxia) [13], and apoptosis [8,17]. Immunoelectron studies of cells arrested in mitosis showed the presence of proteasomes around the chromosomes [15], while others showed the association of proteasome with the spindle poles in metaphase [1]. Changes in proteasome distribution have also been observed in cells during apoptosis. For example, proteasomes have been detected in the apoptotic blebs in ovarian granulose cells [17] and lung cancer cell line [8]. Translocation of the proteasomes into the nucleus has also been reported [13]. Ogiso et al. [13] showed increased proteasomal distribution in the nuclei of glucose-starved cells and hypoxic cancer cells. Based on the above studies, our results could reflect certain stress conditions in PD that result in altered distribution of proteasome in the neuronal cell nuclei.

In this regard, McNaught et al. [9] used immunohistochemistry and Western blotting to describe selective reduction of α-subunits in dopaminergic neurons. Their finding is in sharp contrast to the result of the present study in SN. The reason for the different results in the two studies is not clear at present. However, it may be due to the different methods used in the analysis including the antibodies.

Regarding the activities of 20S proteasome, there are controversies about whether or not the activities are decreased in PD. McNaught et al. [10] reported moderately reduced peptidase activity of the proteasome in idiopathic PD. However, it is