

and exon–intron boundaries and gene dosages of neurodegenerative disease-related genes.

We selected 19 genes (*SOD2*, *SOD3*, *ALS2/alsin*, *SMN1*, *PGRN*, *ANG*, *VEGF*, *VCP*, *VAPB*, *DCTN1*, *CHMP2B*, *TDP-43*, *GSK3 β* , *alpha-synuclein*, *LRRK2*, *parkin*, *DJ-1*, *PINK1*, and *ATP13A2*) as candidate genes for Kii ALS/PDC in this study. Among these ALS-, FTL-, tauopathy-, and synucleinopathy-related genes, some of the genes might be linked in a common pathway leading to neurodegeneration. With regard to gene–gene and protein–protein interactions, there are many interactions between *GSK3 β* and key components related to neuropathology of AD such as tau. *GSK3 β* phosphorylates tau in the fly model and could modulate τ -induced neurodegeneration or at least widespread NFT formation.⁴⁰ Moreover, phosphorylation of human tau following overexpression of the *Drosophila* *GSK3* homolog Shaggy resulted in the formation of neurofibrillary pathology, including paired helical filaments.⁴⁰ In addition, *LRRK2* was reported to link tauopathy and synucleinopathy.³⁵

We also analyzed gene dosage abnormalities in exons 1, 9, 10, 13 of *MAPT*, exon 3 of *alpha-synuclein*, exon 3 of *TDP-43*, exon 5 of *GSK3 β* , and all exons of *parkin*. Recently, further evidence for causative rearrangements has been presented in synucleinopathy and tauopathy. Indeed, multiplications of *alpha-synuclein* have been reported in PD/diffuse Lewy body disease with gene dosage effect. On the other hand, mouse models that expressed the shortest isoform of tau in a wild-type background acquired age-dependent pathology that was similar to FTDP-17 and Guam ALS/PDC: presence of insoluble, hyperphosphorylated tau and argyrophilic intraneuronal inclusions formed by tau immunoreactive filaments.⁴¹ These were the first transgenic mice to recapitulate key features of human tauopathies associated with motor weakness observed in ALS/PDC.⁴¹

In this study, we showed the absence of causative mutation in all the major 19 related genes examined by direct sequencing and no gene dosage abnormalities in exons 1, 9, 10, 13 of *MAPT*, exon 3 of *alpha-synuclein*, exon 3 of *TDP-43*, exon 5 of *GSK3 β* , and all exons of *parkin*. To clarify the regulatory system for abundant tau deposits, approaches to promoters or intronic regions, other genetic factors that could not be detected by direct sequencing, gene rearrangements, epigenetics, or gene–gene interaction, might also be needed. A recent study reported the deposition of TDP-43 in the Guam PDC⁴² and Kii ALS/PDC (unpublished data by Kuzuhara et al.). Deposition of TDP-43 in addition to tau might be a major feature in ALS/PDC. More recently, mutations of *TDP-43* were

identified to cause familial non-*SOD1* ALS and sporadic ALS.⁴³ Thus, further studies are needed to identify the roles of TDP-43, phosphorylated TDP-43, tau, and phosphorylated tau in ALS/PDC. Although we could not detect multiplication of *MAPT*, *GSK3 β* , and *TDP-43*, overexpression of tau or other genes remains an important issue.

In this study, a homozygous nonsynonymous SNP (*ALS2/alsin* V368M: rs3219156) shared in all three patients is not likely to be a harmful polymorphism because of the high allele frequency in dbSNP database of normal Asian population and in our controls of healthy Japanese population. However, because the phenotype of ALS and PDC is heterogeneous even in the same family, some common genetic factors such as SNPs that are sensitive to certain agents in the early stages of life might be also underlying mechanisms solely or in combination. On the other hand, because rare diseases such as ALS/PDC might be caused by certain rare variants, further association studies including healthy controls in the Kii peninsula would be needed then. Furthermore, although the ethnic background is different, it is intriguing to investigate whether some shared SNPs among patients with the western Pacific ALS/PDC exist or not. Thus, these issues should be investigated thoroughly in western Pacific ALS/PDC.

To date, the etiology of ALS/PDC of the Kii peninsula and Guam remains unclear, and we could not identify any causative mutations of the known genes related to ALS/FTLD, parkinsonism and dementia in our Kii ALS/PDC patients. However, aggregation of the disease in some families and absence of any confirmed environment factors suggest the involvement of other genetic factors in the pathogenesis of ALS/PDC.

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Mutation Analysis of the *PINK1* Gene in 391 Patients With Parkinson Disease

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Objectives: To determine the frequency, distribution, and clinical features of Parkinson disease (PD) with *PINK1* mutations.

Design: Retrospective clinical and genetic review.

Setting: University hospital.

Patients: We performed extensive mutation analyses of *PINK1* in 414 PD patients negative for *parkin* mutations (mean [SD] age at onset, 42.8 [14.3] years), including 391 unrelated patients (190 patients with sporadic PD and 201 probands of patients with familial PD) from 13 countries.

Results: We found 10 patients with PD from 9 families with *PINK1* mutations and identified 7 novel mutations (2 homozygous mutations [p.D297MfsX22 and p.W437R] and 5 single heterozygous mutations [p.A78V, p.P196QfsX25, p.M342V, p.W437R, and p.N542S]). No compound heterozygous mutations were found. The frequency of homozygous mutations was 4.26% (2 of 47) in families with autosomal recessive PD and 0.53% (1 of 190) in patients with

sporadic PD. The frequency of heterozygous mutations was 1.89% (2 of 106) in families with potential autosomal dominant PD and 1.05% (2 of 190) in patients with sporadic PD. The mean (SD) age at onset in patients with single heterozygous mutations (53.6 [11.1] years; range, 39-69 years) was higher than that in patients with homozygous mutations (34.0 [20.3] years; range, 10-55 years). Myocardial iodine-123 metaiodobenzylguanidine uptake was low in patients with heterozygous mutations but not in those with homozygous mutations.

Conclusions: Our results suggest that homozygous *PINK1* mutations tend to be diagnosed as the early-onset autosomal recessive form of PD. Single heterozygous mutations may contribute to the development of sporadic PD and also could be an additional genetic predisposition for developing familial PD. The reduced myocardial iodine-123 metaiodobenzylguanidine uptake observed in patients with single heterozygous *PINK1* mutations is similar to that seen in patients with sporadic PD.

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PARKINSON DISEASE (PD) IS PRE- dominantly characterized by degeneration of midbrain dopaminergic neurons, eventually leading to various motor dysfunctions, such as rigidity, tremor, bradykinesia, and postural instability.¹ The etiology of PD is unknown but is presumably multifactorial, eg, perhaps having a genetic × environmental interaction.

Although most PD cases are sporadic, several causative genes have been identified in recent years in familial forms of PD. For example, *alpha-synuclein* (loci, *PARK1* and *PARK4*), *UCH-L1* (*PARK5*), and *LRRK2/dardarin* (*PARK8*) are reported to be the causative genes for autosomal dominant PD (ADPD)²⁻⁶; and *parkin* (*PARK2*), *DJ-1* (*PARK7*), and *PINK1* (OMIM 608309) (*PARK6*) are reported to be the causative

genes for autosomal recessive PD (ARPD).⁷⁻⁹ Mutations in *parkin* are the major cause of ARPD, and the frequency of such mutations in families with ARPD is approximately 50%.¹⁰ In contrast, mutations in *DJ-1* are rare (≤ 1%) in ARPD.¹¹ Increasing numbers of patients with *PINK1* mutations are being reported; however, there are no sufficiently large studies to define the frequency, age distribution, or clinical features of patients with PD associated with *PINK1* mutations worldwide, especially not in Asia. Moreover, no association between PD and coding single nucleotide polymorphisms within *PINK1* has been reported.¹² The role of a single heterozygous *PINK1* mutation in the clinical manifestation of parkinsonism, such as age at onset, is not clear at present, mainly because previous reports have not identified substantial num-

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Table 1. Characteristics of 414 Analyzed Patients With Parkinson Disease

Type of Disease	No. of Patients	Mean (SD) Age at Onset, Range, y
Sporadic Parkinson disease	190 (105 males, 85 females)	37.2 (10.4), 7-81
Familial Parkinson disease	224 (201 probands, 23 relatives; 100 males, 124 females)	47.6 (15.5), 10-85
ARPD	55 (47 probands)	52.8 (13.8)
ADPD	121 (106 probands)	47.1 (15.8)
Unclear hereditary information	48	43.1 (16.0)
Total	414 (391 unrelated patients [190 patients with sporadic disease and 201 probands])	42.8 (14.3)

Abbreviations: ADPD, autosomal dominant Parkinson disease; ARPD, autosomal recessive Parkinson disease.

bers of *PINK1* mutations. To clarify these aspects, we performed extensive mutation analysis in a large number of patients with PD in 13 countries.

METHODS

PATIENTS

We studied 414 *parkin*-negative PD patients (391 unrelated patients and 23 relatives) from 13 countries (249 Japanese, 55 Korean, 28 Israeli, 27 Taiwanese, 27 Chinese, 14 Tunisian, 5 Turkish, 3 Greek, 2 Moroccan, 1 Filipino, 1 Bulgarian, 1 Brazilian, and 1 Australian individual). Patients received clinical diagnoses of PD¹³ regardless of their familial history. The distribution of age at onset was as follows: younger than 50 years (early-onset) (n = 287 [69.3%]), 50 years or older (late-onset) (n = 117 [28.3%]), and unknown (n = 10 [2.4%]). Hereditary information and the mean ages at onset of patients with PD are provided in **Table 1**. In this study, we defined mode of inheritance as autosomal recessive (≥ 2 affected siblings in only 1 generation) and autosomal dominant (≥ 1 affected member in 2 consecutive generations). All participants in the control cohort were Japanese individuals. The study was approved by the ethics review committee of Juntendo University. Blood samples for genetic analysis and clinical information were collected by local neurologists after obtaining informed consent from the patients.

GENETIC ANALYSIS

Genomic DNA was isolated from peripheral blood using standard protocols. For direct sequence analysis, DNA was amplified by polymerase chain reaction of each exon, using standard methods and published primers.¹⁴ Dideoxy sequencing was performed with Big Dye Terminator Chemistry (Applied Biosystems, Foster City, California). These products were loaded on ABI 377, ABI 310, and ABI 3130 automated DNA sequence analyzers (Applied Biosystems) and analyzed with DNA Sequence Analysis software (Applied Biosystems). *Parkin* mutations were examined by polymerase chain reaction, direct sequencing, and quantitative assays based on real-time polymerase chain reaction with TaqMan probes (Applied Biosystems) in each exon. We ruled out *parkin* mutations including exonic deletions or multiplications by dosage studies before analysis of *PINK1*. For extensive screening of substitutions and to determine whether or not the novel *PINK1* mutations were pathogenic, we performed direct sequencing in 300 chromosomes of healthy control DNA samples for all coding exons. All controls were evaluated by neurologists to ensure none of them had parkinsonism.

GENE DOSAGE STUDIES

We performed polymerase chain reaction–based exon dosage assay using TaqMan Chemistry and an ABI PRISM 7700 sequence detection system (Applied Biosystems) in the 5 patients who had a heterozygous *PINK1* mutation (patients E, F, G, H, and J) to rule out compound heterozygous mutations with other heterozygous exonic deletion or multiplication. We used the primer and the probe of Assay by Design (Applied Biosystems) according to a previously published report.¹⁴

MYOCARDIAL IODINE-123 METAIODOBENZYLGUANIDINE SCINTIGRAPHY

Myocardial iodine-123 metaiodobenzylguanidine (¹²³I-MIBG) scintigraphy was performed in 5 *PINK1* mutation–positive patients (from different hospitals) with an intravenous injection of 111 MBq of ¹²³I-MIBG (Daiichi Radioisotope Laboratories, Tokyo, Japan). Early images were obtained 15 minutes and delayed images were obtained 3 to 4 hours after injection. Whole myocardial ¹²³I-MIBG uptake was measured on a planar image as the early and delayed heart to mediastinum activity ratio.

STATISTICAL ANALYSIS

Data are expressed as mean (SD). For continuous variables, such as age at onset, the *t* test was used to test for significant differences between the 2 groups. Categorical data, such as individual responses to each question on the diagnosis checklist and frequencies, were compared with the χ^2 test, with Yates correction when appropriate.

RESULTS

GENETIC ANALYSIS

We identified 10 patients with PD from 9 families with *PINK1* mutations, including 7 novel mutations (**Table 2**). Three homozygous missense mutations were found in 4 patients: p.T313M, p.C388R, and a novel p.W437R. Previously, p.T313M and p.C388R had been reported.^{15,16} In addition, 3 novel single heterozygous missense mutations were found in 4 patients: p.A78V, p.M342V, and p.N542S. We also identified 1 novel homozygous deletion (p.D297MfsX22) and 1 novel single heterozygous deletion (p.P196QfsX25). We also found 1 patient with familial PD (without clear mode of inheritance) with a novel single heterozygous variant (p.V482M).

Table 2. Clinical Features of Study Patients With *PINK1* Mutations

Measure	Missense Mutations								Homozygous Deletion	Heterozygous Deletion
	Homozygous				Heterozygous					
Patient	A	B	C	D	E	F	G	H	I	J
Nucleotide change	c.938C>T	c.1162T>C	c.1162T>C	c.1309T>C	c.233C>T	c.1024A>G	c.1024A>G	c.1625A>G	c.889delG	c.585delC
Amino acid change	p.T313M	p.C388R	p.C388R	p.W437R	p.A78V	p.M342V	p.M342V	p.N542S	p.D297MfsX22	p.P196QfsX25
Exon	4	6	6	7	1	5	5	8	4	2
Hereditary form	SPD	ARPD	ARPD	FPD	SPD	SPD	ADPD	ADPD	ARPD	FPD
Country of residence	Japan	Japan	Japan	Turkey	Japan	Japan	Japan	Japan	Greece	Japan
Consanguinity	-	+	+	+	-	-	-	-	+	+
Age at onset, y	32	55	54	19	39	49	53	69	10	58
Disease duration, y	2	10	2	21	21	12	4	7	15	16
Sex	M	F	F	F	F	F	M	F	F	F
Resting tremor	+	+	+	+	+	+	+	+	+	+
Rigidity	+	+	+	+	+	+	+	+	+	+
Bradykinesia	-	-	-	+	+	+	+	+	+	+
Postural instability	-	-	-	-	+	+	+	-	+	-
Gait disturbance	+	+	+	+	+	+	+	+	+	+
Frozen gait	+	-	-	+	-	-	-	-	+	+
Wearing off	+	-	-	+	-	+	-	+	+	-
On/off states	+	-	-	+	-	+	-	-	+	NA
Asymmetry at onset	-	-	+	+	-	+	-	+	-	+
Orthostatic hypotension	-	-	-	-	+	-	-	-	-	+
Incontinence	-	-	+	-	-	-	-	+	-	+
Urinary urgency	+	-	-	+	-	+	-	+	-	-
Levodopa-induced dyskinesia	-	-	-	+	-	+	-	-	+	-
Sleep benefit	-	-	+	-	-	-	-	-	-	-
Dystonia at onset	-	-	-	-	-	-	-	-	+	-
Hyperreflexia	-	-	-	-	-	-	-	-	+	+
Dementia	-	-	-	-	-	-	-	+	-	-
Depression	-	-	-	-	-	-	-	-	-	-
Hallucinations	-	-	-	-	-	+	-	+	-	-
Other psychosis	-	-	-	-	-	-	-	-	-	-
UPDRS score, on/off states	19/39	18/NA	NA/6	16/22	NA	NA	NA	NA	77/NA	64/259
Hoehn-Yahr stage										
On state	2	2	1	1.5	2.5	4	3	2	2	3
Off state	3	NA	NA	2.5	NA	NA	NA	3	4	NA
Myocardial ¹²³ I-MIBG uptake	NA	Not decreased	Not decreased	NA	Decreased	NA	NA	Decreased	NA	Decreased
Early H:M activity ratio (standard value)	NA	1.82 (> 1.45)	1.97 (> 1.45)	NA	1.49 (> 1.45)	NA	NA	1.4 (> 1.84)	NA	1.64 (> 2.2)
Delayed H:M activity ratio (standard value)	NA	2.93 (> 1.45)	1.97 (> 1.45)	NA	1.25 (> 1.45)	NA	NA	1.18 (> 1.78)	NA	1.28 (> 2.2)

Abbreviations: ADPD, autosomal dominant Parkinson disease; ARPD, autosomal recessive Parkinson disease; FPD, familial Parkinson disease (definite information on mode of inheritance not available, though some family members had parkinsonism); H:M, heart to mediastinum; NA, not applicable or no information available; SPD, sporadic Parkinson disease; UPDRS, Unified Parkinson Disease Rating Scale; +, present; -, absent; ¹²³I-MIBG, iodine-123 metaiodobenzylguanidine.

We did not find any of these mutations or variants in 300 chromosomes in a healthy Japanese population, and we did not detect exonic deletion or multiplication by gene dosage study. The aforementioned novel missense mutations and variant have not been reported as polymorphisms. In addition, we examined the homology regarding the *PINK1* protein. The site of p.W437R mutation was highly conserved among various species. On the other hand, the p.V482M variant was not highly conserved (data not shown).

The affected relatives of patients G, H, and J could not be tested for cosegregation of the same heterozygous mutation that was found in the probands. Thus, we could not exclude that the mutation does not cosegregate in 1 or more of these families. No cosegregation of the p.V482M variant was observed among patients in the same family. Therefore, the role of this variant in this family was not clear.

The frequency of homozygous *PINK1*-positive patients was 1.02% (4 of 391 [1 patient with sporadic PD + 3

familial PD probands]/[190 patients with sporadic PD + 201 familial PD probands]) among the entire group of PD patients. Furthermore, the frequency of homozygous *PINK1*-positive patients was 4.26% (2 of 47) in ARPD families and 0.53% (1 of 190) in patients with sporadic PD. Homozygous mutations were not detected in patients with ADPD. However, the frequency of single heterozygous *PINK1*-positive patients was 1.28% (5 of 391) among the entire group of PD patients, 1.89% (2 of 106) in ADPD families, and 1.05% (2 of 190) among patients with sporadic PD. No single heterozygous mutations were detected in patients with ARPD.

CLINICAL ANALYSIS

Table 2 lists the clinical features of 10 *PINK1*-positive patients and the **Figure** shows the pedigree of families with the *PINK1* mutation. In this study, the family with no cosegregation of p.V482M was excluded from Table 2 and the **Figure**, because the role of the V482M variant in this

family was not clear. Among the *PINK1*-positive families, consanguineous marriages were noted in 5 patients (patients B, C, D [pedigree not available], I, and J).

The mean age at onset of patients with homozygous *PINK1* mutations was 34.0 (20.3) years (range, 10-55 years), and that of patients with a single heterozygous *PINK1* mutation was 53.6 (11.1) years (range, 39-69 years). The age at onset was significantly lower in the homozygous *PINK1*-positive patients compared with the single heterozygous *PINK1*-positive and *PINK1*-negative patients.

As presented in Table 2, motor dysfunction was comparatively mild in many *PINK1*-positive patients. The mean Hoehn-Yahr stage of homozygous *PINK1*-positive patients was 1.7 (0.4) in the on state and 3.3 (0.6) in the off state. In contrast, the average Hoehn-Yahr stage of patients with a single heterozygous *PINK1* mutation was 2.9 (0.5) in the on state and 3.0 (0.0) in the off state. Even in patient E, who had had PD for 21 years, the Hoehn-Yahr stage was 2.5. None of the patients had a Hoehn-Yahr stage of 5.0.

Patient I had a homozygous 1-base deletion mutation and patient J had a single heterozygous 1-base deletion mutation. These 2 patients had similar deletion mutations that caused stop codons within the serine/threonine kinase domain of *PINK1*, but age at onset was clearly different: 58 years for patient J (the latest) and 10 years for patient I (the earliest among *PINK1*-positive patients). Although both patients had hyperreflexia, patient J did not have dystonia at onset, while patient I had dystonia at onset. To date, none of the *PINK1*-positive patients in this study were investigated pathologically.

MYOCARDIAL ¹²³I-MIBG SCINTIGRAPHY

Myocardial ¹²³I-MIBG scintigraphy was performed in 5 *PINK1*-positive patients (patients B, C, E, H, and J). The early and delayed heart to mediastinum ratios of these patients are listed together with the age-matched standard values in Table 2. Myocardial ¹²³I-MIBG uptake was normal in patients with homozygous *PINK1* mutations (patients B and C), whereas it was decreased in patients with single heterozygous *PINK1* mutations (patients E, H, and J).

COMMENT

Combining the results of our previous studies^{14,15,17} and this study, the frequency of *PINK1*-positive families with 2 allele mutations (homozygous mutations and compound heterozygous mutations) among *parkin*-negative ARPD was 11.5% (10 of 87). Among heterozygous mutations, many were single heterozygous rather than compound heterozygous. Our results showed that not only a Japanese individual but 1 Greek and 1 Turkish individual had *PINK1* mutations (Table 2), which suggests that the mutation is possibly distributed worldwide, similar to *parkin* mutations.^{10,11} Considering previous reports on the frequencies of *parkin*^{10,11} and *DJ-1*^{18,19} mutations, we propose that we should first screen patients with PD for *parkin* mutations, including gene dosage

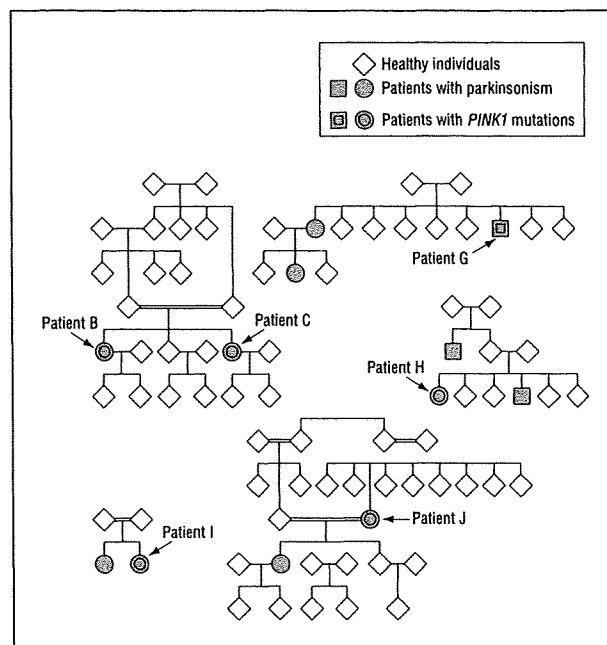


Figure. Pedigrees of patients with *PINK1* mutations. Patients B and C had the same homozygous missense mutation (p.C388R) in exon 6. Patient G had a single heterozygous mutation (p.M342V) in exon 5. Patient H had a single heterozygous mutation (p.N542S) in exon 8. Patient I had a homozygous deletion mutation (p.D297MfsX22) in exon 4, and patient J had a single heterozygous deletion mutation (p.P196QfsX25) in exon 2. The sexes are concealed to safeguard the confidentiality of the family members.

study, then screen for *PINK1* mutations, and finally screen for *DJ-1* in ARPD.

In the present study, we did not screen fully for heterozygous *PINK1* deletion mutations and multiplications by the gene dosage study using TaqMan assay to save time in screening all patients. Homozygous *PINK1* deletion mutation of more than 1 exon structure had been reported in only 1 case so far.¹⁵ *PINK1* and *DJ-1* deletion mutations seem to be less frequent than *parkin* deletion mutations even if these heterozygous deletion mutations are to be included. In this regard, we think that gene dosage study of *PINK1* may not be as important as that of *parkin*.

Although the prevalence was rare, our study and others²⁰⁻²² showed that homozygous mutations as well as single heterozygous *PINK1* mutations are found not only in ARPD but also in ADPD families and patients with sporadic PD. These results suggest that screening for *PINK1* mutations may also be necessary in patients with potential ADPD and sporadic PD.

Although heterozygous carriers are clinically unaffected in most autosomal recessive disorders, higher preponderance of heterozygous *PINK1* mutations in patients with sporadic PD, compared with matched controls, has been reported.²¹⁻²³ Accordingly, although it is difficult to make a firm conclusion about the frequencies of heterozygous *PINK1* mutations in patients vs controls, all the single heterozygous *PINK1* mutations were found only in Japanese patients with PD but not in Japanese controls. Moreover, in the positron-emission tomographic study, carriers of heterozygous *PINK1* mutations showed significant reductions in caudal and putaminal fludeoxyglucose F18 uptake (mean of 20%-30% lower than the controls), indicating increased susceptibility for the de-

Table 3. Clinical Features of 23 Patients With *PINK1* Mutations in Current and Past Studies

Measure	No. of Patients					P Value		
	No <i>PINK1</i> Mutation (n=404)	<i>PINK1</i> -Mutation Positive				2 Mutations vs 1 Mutation	2 Mutations vs No Mutation	Mutation Positive vs No Mutation
		Homozygous (n=16)	Compound Heterozygous (n=2)	Heterozygous (n=5)	All (n=23)			
Sporadic PD	187	1	0	2	3			
ARPD ^a	52	14	2	0	16			
ADPD	119	0	0	3	3			
Patients with familial PD, unclear hereditary information	46	1	0	0	1			
Age at onset, mean (SD), y	42.8 (14.3)	32.6 (8.5)	18.5 (0.7)	53.6 (11.1)	35.9 (15.0)	< .001	< .001	.03
Resting tremor	293	11	2	5	18	.47	.83	.72
Rigidity	366	13	2	5	20	.82	.54	.83
Bradykinesia	368	12	2	5	19	.62	.14	.32
Postural instability	244	9	0	4	13	.49	.53	.88
Gait disturbance	268	15	1	5	21	.91	.08	.02
Frozen gait	NA	9	0	1	10	.49	NA	NA
Wearing off	227	11	2	2	15	.42	.27	.53
On/off states	NA	9	0	1	10	.49	NA	NA
Asymmetry at onset	293	9	1	3	13 ^b	.64	.34	.26
Orthostatic hypotension	43	2	0	2	4 ^c	.57	.99	.30
Incontinence	30	1	0	2	3 ^b	.23	.81	.52
Urinary urgency	63	2	0	2	4 ^b	.44	.93	.98
Levodopa-induced dyskinesia	170	6	2	1	9	.51	.96	.95
Sleep benefit	112	6	2	0	8	.14	.20	.62
Dystonia at onset	53	3	1	0	4	.62	.45	.79
Hyperreflexia	51	8	0	1	9	.50	.001	.001
Dementia	41	2	0	0	2	.91	.79	.90
Depression	NA	2	0	0	2	.91	NA	NA
Hallucinations	62	2	0	2	4	.40	.88	.97
Other psychosis	26	1	0	0	1	.48	.73	.97
Hoehn-Yahr stage, mean (SD)								
On state	2.5 (1.0)	2.3 (0.7)	NA	2.9 (0.5)	2.5 (0.7)	.06	.29	.48
Off state	3.4 (1.1)	2.9 (0.9)	NA	3.0 (0.0)	3.3 (1.1)	NA	.27	.24

Abbreviations: ADPD, autosomal dominant Parkinson disease; ARPD, autosomal recessive Parkinson disease; NA, not applicable; PD, Parkinson disease.

^aThirteen of the patients with ARPD were reported previously by our group.^{13,15,17}

^bn=22.

^cn=19.

velopment of parkinsonism.²⁴ In addition, our data showed that the age at onset of patients with heterozygous *PINK1* mutations was higher than that of patients with homozygous *PINK1* mutations and was similar to that of classic sporadic PD. Thus, the previous findings and our data emphasize the importance of heterozygous *PINK1* mutations as a possible risk factor for developing the common classic form of sporadic PD. However, we could not exclude other possibilities, eg, that these mutations could be coincidental findings or even be a cause of ADPD, because we did not perform the genetic tests in the relatives of the patient with a single heterozygous mutation or in controls outside of the Japanese population. In addition, we could not exclude the possibility of digenic inheritance or technical limitations in detecting all possible mutations (eg, in the introns and promoter).

Table 3 lists the clinical symptoms of the patients in this study and patients reported previously by our group.^{14,15,17} Thus, we could compare 23 *PINK1*-positive patients with 404 *PINK1*-negative patients and compare 18 patients with 2 allele *PINK1* mutations (16

patients with homozygous *PINK1* mutations and 2 patients with compound heterozygous mutations) with 5 patients with 1 allele *PINK1* mutation. The data in Table 3 show that most *PINK1*-positive patients develop early-onset parkinsonism. Moreover, the mean age at onset of patients with 1 allele *PINK1* mutation was higher than that of patients with 2 allele mutations.

Age at onset, hyperreflexia, and gait disturbances were significantly more frequent in homozygous *PINK1*-positive patients than in *PINK1*-negative patients. Indeed, these symptoms were also significantly different in patients with or without *PINK1* mutations. However, there were no statistical differences in pathognomonic symptoms between patients with 1 or 2 allele *PINK1* mutations, except for age at onset. These data indicate that the phenotypes of patients with a single heterozygous *PINK1* mutation are more likely to be similar to those of homozygous *PINK1*-positive patients, except for age at onset.

Myocardial ¹²³I-MIBG scintigraphy is one of the most supportive diagnostic tools used in differentiating PD from

conditions such as essential tremor, progressive supranuclear palsy, and multiple system atrophy.^{25,26} In this regard, some patients with 2 allele *parkin* mutations without Lewy bodies were reported to have normal ¹²³I-MIBG uptake.²⁷⁻²⁹ Another study demonstrated markedly low heart to mediastinum ratios in patients with classic PD with Lewy bodies and in incidental Lewy body disease, suggesting that Lewy body pathology itself may be responsible for low ¹²³I-MIBG uptake.³⁰ Although a single case with a homozygous *PINK1* mutation was reported to have a very mild decrease in ¹²³I-MIBG uptake,³¹ our data showed that 2 patients with homozygous *PINK1* mutations (patient B with disease duration of 10 years and patient C with disease duration of 2 years) had normal myocardial ¹²³I-MIBG uptake. In contrast, 3 patients with single heterozygous *PINK1* mutations (patients E, H, and J) had low myocardial ¹²³I-MIBG uptake. These findings suggest that patients with a single heterozygous mutation are more likely to have cardiac sympathetic denervation than those with homozygous *PINK1* mutations, which accounts for the low ¹²³I-MIBG uptake. One can further speculate that patients with heterozygous *PINK1* mutations may have Lewy body pathology, whereas those with homozygous *PINK1* mutations have no Lewy body pathology, similar to patients with *parkin* mutations,^{10,32} though no pathologic study of patients with 2 allele *PINK1* mutations has been reported to date. Additional studies of cardiac scintigraphy in a larger number of *PINK1*-positive patients with PD are required to clarify these points.

In summary, we assume that homozygous *PINK1* mutations may manifest in an early-onset autosomal recessive form of PD. We can also speculate that single heterozygous mutations may be 1 of the risk factors in developing the sporadic or autosomal dominant form of PD. Additional studies are necessary to clarify the etiological roles of 1 allele *PINK1* mutation in developing various forms of PD.

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Calbindin 1, fibroblast growth factor 20, and α -synuclein in sporadic Parkinson's disease

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Abstract Parkinson's disease (PD), one of the most common human neurodegenerative disorders, is characterized by the loss of dopaminergic neurons in the substantia nigra of the midbrain. Our recent case-control association study of 268 SNPs in 121 candidate genes identified α -synuclein (*SNCA*) as a susceptibility gene for sporadic PD ($P = 1.7 \times 10^{-11}$). We also replicated the association of *fibroblast growth factor 20* (*FGF20*) with PD ($P = 0.0089$). To find other susceptibility genes, we added 34 SNPs to the previous screen. Of 302 SNPs in a total 137 genes, but

excluding *SNCA*, SNPs in *NDUFV2*, *FGF2*, *CALB1* and *B2M* showed significant association ($P < 0.01$; 882 cases and 938 control subjects). We replicated the association analysis for these SNPs in a second independent sample set (521 cases and 1,003 control subjects). One SNP, rs1805874 in *calbindin 1* (*CALB1*), showed significance in both analyses ($P = 7.1 \times 10^{-5}$; recessive model). When the analysis was stratified relative to the *SNCA* genotype, the odds ratio of *CALB1* tended to increase according to the number of protective alleles in *SNCA*. In contrast, *FGF20* was significant only in the subgroup of *SNCA* homozygote of risk allele. *CALB1* is a calcium-binding protein that widely is expressed in neurons. A relative sparing of

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CALB1-positive dopaminergic neurons is observed in PD brains, compared with CALB1-negative neurons. Our genetic analysis suggests that *CALB1* is associated with PD independently of *SNCA*, and that *FGF20* is associated with PD synergistically with *SNCA*.

Introduction

Parkinson's disease (PD) (OMIM 168600), which affects one to two percent of people age 65 or older (de Rijk et al. 1997) is one of the most common human neurodegenerative diseases, second in incidence only to Alzheimer's disease (OMIM 104300). Clinical features of PD include resting tremor, bradykinesia, rigidity and postural instability. PD is characterized pathologically by the loss of dopaminergic neurons in the substantia nigra of the midbrain and by the presence of intracellular inclusions known as Lewy bodies (Shults 2006). Various types of medical management are available for PD, including drugs (L-Dopa, dopamine agonists, anti-cholinergic drugs and others) and surgery (e.g., thalamotomy, pallidotomy, deep brain stimulation) (Rascol et al. 2003). These treatments improve PD symptoms but do little to deter disease progression. Identifying risk factors for PD can thus be helpful in delaying disease onset and slowing its progression.

Genetic approaches for Mendelian-inherited PD have identified autosomal dominant genes including *α-synuclein* (*SNCA*) and *LRRK2*, as well as autosomal recessive genes *parkin*, *PINK1*, *DJ-1*, and *ATP13A2* (Thomas and Beal 2007).

However, Mendelian-inherited PD is rare compared with the far more common sporadic PD, a complex disorder caused by multiple genetic and environmental factors (Warner and Schapira 2003). Using a multiple candidate gene analysis, we previously identified and confirmed *SNCA* (4q21) as a definite susceptibility gene for sporadic PD (Mizuta et al. 2006). In addition we recently replicated the significance of *FGF20* (8p22-p21.3) (Satake et al. 2007). Here, we have found a novel PD susceptibility gene *calbindin 1* (*CALB1*, 8q21.3-q22.1) from multiple candidate gene analysis.

Methods

Subjects

We recruited two independent sample sets, comprised of individuals with Japanese ancestry. Sample Set 1, described in our previous report (Mizuta et al. 2006), included 882 unrelated sporadic PD patients (age = 64.9 ± 9.8 ; male/female ratio = 0.79; onset = 57.4 ± 10.9 years of age; 51

patients with a positive family history) and 938 unrelated controls without neurological disorders (age = 45.3 ± 16.3 ; male/female ratio = 1.10). Another independent set (Sample Set 2) consisted of 521 PD patients (age = 67.2 ± 9.7 ; male/female ratio = 0.87; onset = 58.8 ± 11.4 years of age; no family history) and 1,003 control subjects (106 individuals age = 58.9 ± 11.4 ; male/female ratio = 0.86; plus 897 age- and sex-unknown adult subjects). Diagnosis of sporadic PD was based on the presence of two or more of the cardinal features of PD (tremor, rigidity, bradykinesia, and postural instability), determined according to established criteria (Bower et al. 1999). Control subjects are healthy volunteers including spouses of patients. Informed consent was obtained from each participant, and approval for the study was obtained from the University Ethical Committees.

SNP genotyping

Genomic DNA was extracted from whole blood using Flex-Gene (QIAGEN). One hundred and fifteen samples treated subjected to whole genome amplification (GenomiPhi DNA Amplification Kit, GE Healthcare, Buckinghamshire, UK) were included in Sample Set 2. We genotyped the SNPs using the Invader assay (Third Wave Technologies) or TaqMan (Applied Biosystems).

Gene and SNP selection

We selected candidate genes from published reports describing genetic, pathological and biochemical findings in PD, as well as genes that participate in proposed mechanisms for PD. Finally, we included 137 genes relevant to familial PD, Lewy bodies, dopaminergic neurons, cytokines and trophic factors, mitochondrial functions, oxidative stress, proteasome function, autophagy, endoplasmic reticulum-associated degradation (ERAD) and toxins. One to seven SNPs per gene (302 SNPs total) were selected from the dbSNP and JSNP (Haga et al. 2002) databases for analysis. Of 302 SNPs in 137 genes, 268 SNPs in 121 genes had been described elsewhere (Mizuta et al. 2006).

For linkage disequilibrium (LD) analysis, information about chromosomal structure and recombination hotspots was obtained from the HapMap database (<http://www.hapmap.org/>) (The International HapMap Consortium 2005). Japanese tag SNPs (MAF > 0.1, $r^2 > 0.8$) were selected from the HapMap SNP pool using the ABI SNP-browser (<http://www.allsnps.com/snpbrowser>). Typing data for 785 control subjects were used in LD analysis.

Statistical analysis

SNPAlyze software (DYNACOM, Japan) was used for case-control study χ^2 test, haplotype analysis (Expectation-

Maximization algorithm) and pairwise LD analysis (Lewontin's coefficient D' , and standardized coefficient r).

Results

This current study follows our closely related study where 268 SNPs were screened in 121 candidate genes using a case-control analysis. For the current analysis, we examined 34 additional SNPs in 17 candidate genes using a subset of Sample Set 1 (190 cases and 190 control subjects). Five SNPs of them were significant (Supplementary Table 1). Twenty-two significant SNPs from the first study and these five SNPs were then evaluated in all subjects in Sample Set 1 (882 cases and 935 control subjects). In total, this enhanced screen revealed 27 significant SNPs through genotype analysis of 302 SNPs in 137 candidate genes. (One gene, *IL1B*, was included in both the original and the enhanced screen.) Of these 27, the most significant SNP, rs7684318 in *SNCA*, had been reported previously, further confirming *SNCA* as a susceptibility gene for PD (Mizuta et al. 2006). Of the remaining 26 SNPs (Supplementary Table 2), four SNPs in *NDUFV2* (OMIM 600532), *FGF2* (OMIM 134920), *CALB1* (OMIM 114050) and *B2M* (OMIM 109700) showed P -values less than 0.01. They were prompted to genotyping in a second sample set (Sample Set 2) composed of 521 cases and 1,003 control subjects (Supplementary Table 2). *SNCA* rs7684318 was included in this replication study as a quality control to assure that the genotyping was consistent. After application of a Bonferroni correction for 302 SNPs ($\alpha = 1.6 \times 10^{-4}$), the association of rs1805874 in *CALB1* (8q21.3-q22.1) remained significant ($P = 7.1 \times 10^{-5}$; recessive model). The prominent association of *SNCA* rs7684318 ($P = 5.1 \times 10^{-14}$ for allele frequency) was again confirmed (Table 1).

For LD mapping, we genotyped Sample Set 1 using 10 tag SNPs selected from a 230-kb region that surrounds *CALB1* and falls between recombination hotspots (Fig. 1; Table 2). Since most of the pairwise D' values were greater than 0.9, this region is thought to be a single LD block (Fig. 1). Of the ten tag SNPs, rs1805874 and rs1805868 showed significant association with sporadic PD (Table 2). A replication study of the two SNPs using Sample Set 2 confirmed the significance of rs1805874 (Table 2). The haplotype did not show stronger association than the single SNP (Supplementary Table 3). The rs1805874-tagged SNPs are concentrated in and upstream of *CALB1*. The SNPs tagged by closely neighboring ones showing no association span outside of rs1805874-tagged SNPs. This suggests that PD-associated region is restricted in *CALB1* region (Supplementary Fig., Table 4).

Table 1 Association analysis of *CALB1* and *SNCA*

SNP (gene)	Allele (M/m) ^a	Genotype (MM/Mm/mm)		M frequency		P		HWE		
		Case	Control	Case/control	Genotype	Allele	MM + Mm versus mm	MM versus Mm + mm	Case/control	
										MM versus mm
rs1805874 AC (<i>CALB1</i>)		Sample Set 1	549/253/32	538/319/58	0.810/0.762	0.0032	6.1×10^{-4}	0.018	0.0025	0.67/0.25
		Sample Set 2	341/148/23	584/351/43	0.811/0.776	0.023	0.03	0.94	0.0089	0.18/0.28
		Combined	890/401/55	1,121/670/101	0.810/0.770	3.3×10^{-4}	8.5×10^{-5}	0.1	7.1×10^{-5}	0.25/0.95
rs7684318 CT (<i>SNCA</i>)		OR ^b (95% CI)			MM versus mm	Mm versus mm	M versus m	MM + Mm versus mm	MM versus Mm + mm	
		Sample Set 1	385/394/89	295/472/165	1.46 (1.04–2.05)	1.1 (0.77–1.56)	1.28 (1.13–1.44)	1.32 (0.95–1.85)	1.34 (1.16–1.55)	0.42/0.31
		Sample Set 2	213/226/63	323/456/185	0.671/0.570	2.7×10^{-9}	5.0×10^{-10}	5.7×10^{-6}	2.8×10^{-8}	0.80/0.29
		Combined	598/620/152	618/928/350	0.649/0.572	3.1×10^{-4}	4.5×10^{-5}	0.0013	7.6×10^{-4}	0.65/0.96
		OR ^b (95% CI)			MM versus mm	Mm versus mm	M versus m	MM + Mm versus mm	MM versus Mm + mm	
			2.23 (1.79–2.78)	1.54 (1.24–1.91)	1.48 (1.34–1.64)	1.81 (1.48–2.23)	1.6 (1.39–1.85)			

^a Disease allele (M) and protective allele (m)

^b Odds ratio (OR) was calculated in combined sample set

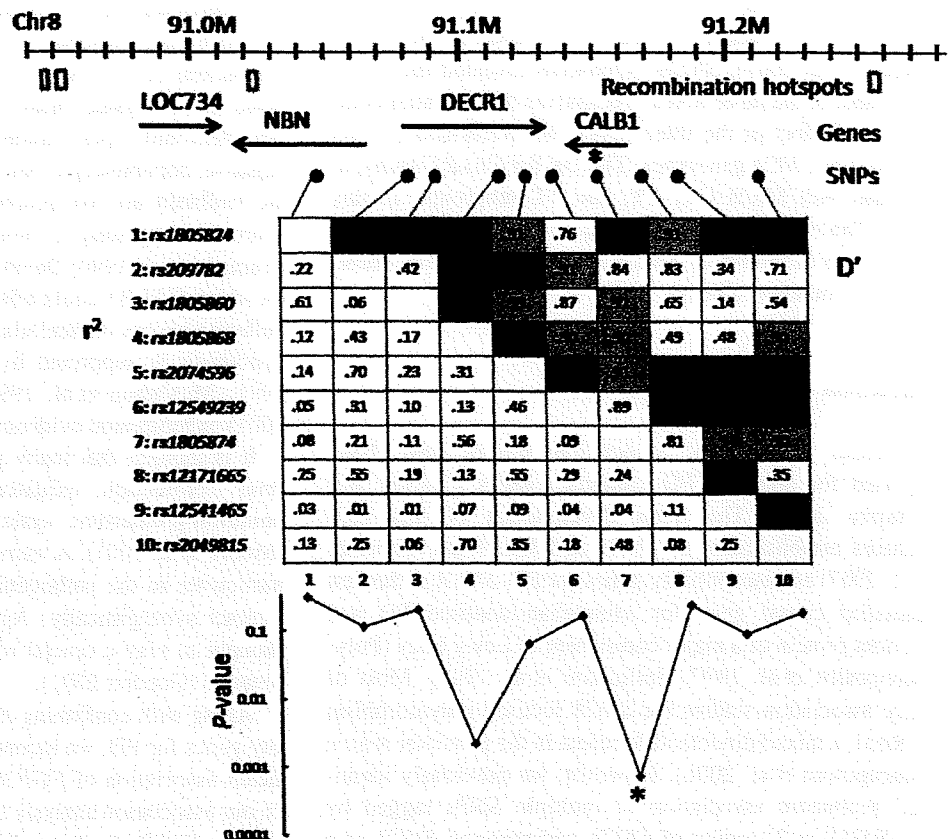
Table 2 Association analysis of SNPs in *CALB1* and the surrounding region

SNP	Location	Allele ^a	Genotype (11/12/22)		MAF ^b	P	HWE		Case/control	
			Case	Control			Case/Control	Genotype Allele		11 + 12 versus 22
Sample Set 1										
rs1805824	<i>NBN</i> (intron)	GA	533/274/53	577/269/51	0.221/0.207	0.59	0.31	0.67	0.31	0.029/0.010
rs2097825	Intergenic	GC	236/386/221	208/416/242	0.509/0.480	0.17	0.10	0.42	0.061	0.015/0.027
rs1805860	<i>DECRI</i> (intron)	GA	431/337/89	460/338/77	0.300/0.281	0.44	0.21	0.26	0.34	0.059/0.19
rs1805868	<i>DECRI</i> (intron)	GA	448/341/63	423/378/103	0.274/0.323	0.0047	0.0015	0.0042	0.015	0.86/0.19
rs2074596	<i>DECRI</i> (intron)	GA	300/369/180	327/416/145	0.429/0.398	0.032	0.057	0.009	0.52	0.0010/0.51
rs12549239	<i>CALB1</i> (3' flanking)	TC	76/329/461	59/348/502	0.278/0.256	0.18	0.15	0.40	0.069	0.12/0.90
rs1805874	<i>CALB1</i> (intron)	CA	32/253/549	58/319/538	0.190/0.238	0.0032	0.00061	0.0025	0.018	0.67/0.25
rs12171665	<i>CALB1</i> (5' flanking)	GA	169/404/280	189/448/271	0.435/0.455	0.40	0.23	0.18	0.60	0.29/0.88
rs12541465		TG	617/210/15	690/185/18	0.143/0.124	0.109	0.103	0.72	0.054	0.55/0.18
rs2049815		TG	378/374/94	382/381/124	0.332/0.355	0.20	0.16	0.072	0.50	0.92/0.067
Sample Set 2										
rs1805868	<i>DECRI</i> (intron)	GA	279/189/44	468/412/83	0.271/0.300	0.077	0.092	0.99	0.031	0.14/0.57
rs1805874	<i>CALB1</i> (intron)	CA	23/148/341	43/351/583	0.189/0.224	0.023	0.030	0.0089	0.94	0.18/0.28

^a Relative to the chromosomal orientation

^b Minor allele frequency

Fig. 1 LD structure and significance of association in the susceptibility region for sporadic PD. *Top* the genomic structure of the *CALB1* region, including genes (arrows) and recombination hotspots (red-lined rectangles), generated from HapMap. Ten SNPs were plotted (closed circles), including the originally screened rs1805874 (marked by an asterisk). *Middle* Pairwise D' (upper right) and r^2 (lower left) in 785 control subjects. *Highlighted cells* contain LD values >0.95 (red) or >0.9 (pink). *Bottom* case-control association studies in Sample Set 1 (882 cases and 938 control subjects). Log P -values (allele 1 vs allele 2) are plotted against the nominal location of the SNPs. The originally screened rs1805874 was indicated by an asterisk



Detailed association analysis in the combined sample sets (Table 1) showed the strongest association for *CALB1* rs1805874 in a recessive model ($P = 7.1 \times 10^{-5}$, OR = 1.34).

The effect of *SNCA* rs7684318 (allele OR = 1.48; genotype OR = 2.23 for the CC genotype and 1.54 for CT genotype) was well described using a multiplicative model (Table 1).

Table 3 Association analysis of *CALB1* and *FGF20*, stratified by *SNCA* genotype

	Case		Control		OR (95% CI)	P
	AA	AC + CC	AA	AC + CC		
<i>CALB1</i> (rs1805874)						
<i>SNCA</i> (rs7684318)						
CC	381	205	381	227	1.11 (0.87–1.40)	0.4
CT	393	203	531	386	1.41 (1.14–1.74)	0.0017
TT	102	44	201	147	1.70 (1.12–2.56)	0.012
<i>FGF20</i> (rs1721100)						
<i>SNCA</i> (rs7684318)						
CC	225	360	159	448	1.76 (1.38–2.25)	5.9×10^{-6}
CT	178	429	281	629	0.93 (0.74–1.16)	0.52
TT	51	98	96	247	1.34 (0.89–2.02)	0.16

OR and *P*-values for 2 × 2 contingency table of *CALB1* and *FGF20* were calculated in *SNCA* CC, CT, and TT genotype subgroups

FGF20 rs1721100, as we reported previously, was most significant in a recessive model ($P = 0.0053$, OR = 1.24) (Satake et al. 2007). *SNCA* rs7684318 revealed the strongest effect of the three SNPs. To analyze the potential combinational effect of the three SNPs, we performed χ^2 tests stratified by *SNCA* genotypes. The OR for *CALB1* tended to increase with *SNCA* CC, CT, and TT subgroups, in this order (Table 3). In contrast, *FGF20* rs1721100 was significant in the subgroup of *SNCA* homozygote of risk allele, but not in others (Table 3).

Discussion

Numerous case-control association studies have been reported for sporadic PD candidate genes (Warner and Schapira 2003). The main development of this field includes establishment of *SNCA* and *MAPT/tau* (Zabetian et al. 2007) as susceptibility genes for PD. *SNCA* is the first identified causal gene for Mendelian-inherited PD and encodes protein of a major component of Lewy body (Polymeropoulos et al. 1997; Spillantini et al. 1997). Most of early association studies for *SNCA* focused polymorphism of Rep1, a mixed dinucleotide repeat in the promoter region (Maraganore et al. 2006). However, we previously identified prominent association in multiple SNPs tagged by rs7684318 in 3' region of *SNCA* and reported *SNCA* as a definite susceptibility gene for sporadic PD (Mizuta et al. 2006). Although rs7684318 is rare in Caucasians, rs356165, tagged by rs7684318 in Japanese (Mizuta et al. 2006), was included in associated SNPs in German study

(Mueller et al. 2005) and very recent Norwegian study (Myhre et al. 2008).

This current report extends our analysis, identifying and confirming *CALB1* as a novel susceptibility gene for this disorder. We found that *CALB1* rs1805874 was significantly associated with PD in Japanese population. The final *P*-value for the association of rs1805874 can be calculated as 2.2×10^{-5} by multiplying the *P*-values of the two independent tests ($P = 0.0025$ for sample set 1 and $P = 0.0089$ for Sample Set 2). This remains significant when Bonferroni correction was applied by multiplying the number of SNPs screened (302 SNPs) and the number of contingency tables per SNP (allele, genotype, recessive model, and dominant model) (the corrected $P = 0.027$). However, this SNP was not significant in previous genome-wide association study in Caucasians (Maraganore et al. 2005). Possible explanations of this discrepancy may include ethnicity and gene-environmental effect.

CALB1 is a 28 kDa protein containing 261 amino acids, originally described as a vitamin D-dependent Ca^{2+} -binding protein in the chick duodenum (Wasserman et al. 1968). Along with calmodulin and troponin C, *CALB1* belongs to the superfamily of Ca^{2+} -binding proteins, which are characterized by the presence of an EF-hand Ca^{2+} -binding loop (Persechini et al. 1989). Though *CALB1* is widely distributed in mammalian brains, it localizes within certain specific neuronal types (Jande et al. 1981). In PD, *CALB1*-negative dopaminergic neurons in the substantia nigra of the midbrain are lost preferentially over *CALB1*-positive neurons, suggesting a neuroprotective role for *CALB1* (Yamada et al. 1990; Damier et al. 1999). One mechanism by which *CALB1* could affect neuronal viability is through buffering excess intracellular Ca^{2+} (Chard et al. 1993). This hypothesis is supported by both in vitro (Mattson et al. 1991; McMahon et al. 1998) and in vivo (Yenari et al. 2001) experimental evidence.

It is thought that many pathways, including mitochondrial dysfunction, oxidative stress, and impairment of ubiquitin-proteasome system, underlie PD pathogenesis (Moore et al. 2005). A number of molecules are thought to participate in the pathogenic process, some of which can interact synergistically. Aggregation of *SNCA* protein is thought to play a crucial role in the loss of dopaminergic neurons (Goedert 2001).

Along with confirming *SNCA* and *CALB1* as susceptibility genes for PD, we recently reported replication of significant association of *FGF20* with PD (Satake et al. 2007). In our association analysis stratified by *SNCA* genotype, the OR for *CALB1* tended to increase according to the number of *SNCA* protective alleles, suggesting the possibility of a negative statistical interaction between *CALB1* and *SNCA*. In contrast, *FGF20* revealed significance only in *SNCA* CC, homozygote of disease allele, suggesting the possibility of

a synergistic statistical interaction between *FGF20* and *SNCA*. It is of interest because *FGF20* risk allele is recently reported to be correlated with high expression of *SNCA* (Wang et al. 2008).

Sporadic PD is a complex multigenic disorder. Combinational analysis of PD susceptibility genes is helpful to evaluate effect of each gene and to uncover pathophysiological mechanism of the disease.

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LRRK2 P755L variant in sporadic Parkinson's disease

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Abstract Parkinson's disease (PD) is a neurodegenerative disorder of unknown etiology with probable involvement of genetic-environmental factors. The majority of PD cases (approximately 90–95%) are sporadic, while familial cases account for approximately 5–10% of PD. In a recent report, a heterozygous *LRRK2* P755L mutation within *LRRK2* exon 19 was found in 2% of Chinese sporadic PD patients and in 0% of normal controls or Caucasians, suggesting that the mutation is disease-associated with ethnic specificity. To further evaluate the role of *LRRK2* P755L variant in sporadic PD, we performed direct sequencing of *LRRK2* exon 19 in

501 Japanese sporadic PD patients (male 249, female 252, aged 28–92 years, mean 65.0 years) and 583 controls of the Japanese general population as an extended association study. In this group, we found six patients (6/501 = 1.2%) and eight controls of the general population (8/583 = 1.6%) with a heterozygous P755L variant ($P = 0.80$, $\chi^2 = 0.064$). No other variants were found in exon 19. Together with previous reports, our extended case-controlled study of large sample size suggests that *LRRK2* P755L is a non-disease-associated polymorphism in PD patients.

Keywords Parkinson's disease · Genetics · *PARK8* · *Leucine-rich repeat kinase 2 (LRRK2)* · Polymorphism · Association study · Japanese · Ethnic background

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Introduction

Parkinson's disease (PD, OMIM #168600) is the second most common neurodegenerative disorder next to Alzheimer's disease. The clinical features are characterized by levodopa-responsive parkinsonism, such as rigidity, resting tremor, bradykinesia, and postural instability. Although the cause of PD remains unclear, genetic-environmental interaction is suggested for the development of the disease. One of the autosomal-dominant forms of PD, *PARK8*, was originally mapped from a Japanese Sagami-hara family (Funayama et al. 2002) and *LRRK2* (*PARK8*; *leucine-rich repeat kinase 2*, OMIM #609007) was identified as the causative gene for *PARK8*-linked PD (Paisán-Ruiz et al. 2004; Zimprich et al. 2004). Among *LRRK2* mutations, the most common *LRRK2* G2019S mutation in North Africans and Ashkenazi Jews has shown ethnic differences among Caucasian, Japanese, and Chinese (Nichols et al. 2005; Gilks et al. 2005; Lesage et al. 2006; Tomiyama et al.

2006; Tan et al. 2005). On the other hand, *LRRK2* G2385R variant has recently been found the most common genetic risk factor among Chinese and Japanese, but not Caucasians (Di Fonzo et al. 2006; Funayama et al. 2007; Tan et al. 2007; Farrer et al. 2007). Moreover, in a recent report (Wu et al. 2006), a heterozygous *LRRK2* p.P755L (c.2264c > t, rs34410987) mutation within *LRRK2* exon 19, corresponding to a predicted ankyrin-repeat-like domain of *LRRK2*, was found in 2% (12/598) of Chinese sporadic PD and 0% (0/765) of Chinese normal controls, suggesting its association with the disease. However, *LRRK2* P755L was reported as a polymorphism (3% of 92 normal controls) in the dbSNP database of Taiwanese. Thus, to determine the frequency and the role of *LRRK2* P755L in Asian PD, we screened for *LRRK2* exon 19 in Japanese sporadic PD patients.

Subjects and methods

The nucleotide sequences of *LRRK2* exon 19 were determined by direct sequencing in 501 sporadic Japanese PD patients and 583 controls of the Japanese general population (Table 1). All blood samples and clinical information were obtained by the attending neurologists after obtaining informed consent from their patients. The study was approved by the ethics review committees of Juntendo and Osaka Universities. Diagnosis of PD was made by the attending neurologists based on the presence of parkinsonism and good response to anti-PD treatment. Controls of the Japanese general population were evaluated by neurologists to ensure none of them had PD. DNA was prepared using standard methods. They were amplified by polymerase chain reaction (PCR) of exon 19 and sequenced using BigDye Terminator Chemistry and ABI310 and 3130 Genetic Analyzer (Applied Biosystems, Foster City, CA). Sequences of the primers, conditions of PCR, and conditions of sequencing were based on a previous report (Zimprich et al. 2004).

Results

We found 6 patients (6/501 = 1.2%) and 8 controls of the Japanese general population (8/583 = 1.6%) with a heterozygous P755L variant ($P = 0.80$, odds ratio = 1.15, 95% CI: 0.40–3.32, $\chi^2 = 0.064$) in *LRRK2* exon 19 (Table 2). No other variants were found in exon 19.

Discussion

The purpose of the present study was to clarify the role of an ethnic-specific variant in the causative gene for PD. Although PD is considered a heterogeneous disease with genetic-environmental interaction, some cases certainly exhibit a Mendelian-inherited disease or are associated with strong genetic and ethnic background. Indeed, the reported frequency of *LRRK2* G2385R was higher in Asian sporadic PD patients than in controls (Di Fonzo et al. 2006; Funayama et al. 2007; Tan et al. 2007), although this is not the case in Caucasians. Moreover, Wu et al. (2006) in Nanjing, China, recently reported that a heterozygous *LRRK2* P755L mutation was found in 2% (12/598) of Chinese sporadic PD and 0% (0/765) of normal controls, whereas none (0/463) of the Caucasian PD patients had this mutation (Deng et al. 2007), suggesting ethnic differences, like *LRRK2* G2385R. However, our results of large case-controlled study in Japanese revealed that *LRRK2* P755L is a non-disease associated polymorphism. Consistent with our data, this variant was present at similar frequency in Taiwanese PD patients (7/578 = 0.99%) and Taiwanese normal controls (10/339 = 0.97%) (Di Fonzo et al. 2006). Furthermore, the latest report in the Chinese population in Singapore showed the absence of segregation and association of P755L with PD (case 4/204 = 2.0%, control 6/235 = 2.6%, $P = 0.76$) (Tan et al. 2008). These findings might be based on ethnic or native differences in human migration history or human genetics.

We reported previously that the most common *LRRK2* G2019S mutation in Mendelian-inherited and sporadic PD

Table 1 Profile of analyzed samples in this study

Parameter	Patients	Controls of general population
Total sample, <i>n</i> (%)	501 (100)	583 (100)
Male, <i>n</i> (%)	249 (49.7)	312 (53.5)
Female, <i>n</i> (%)	252 (50.3)	271 (46.5)
Age at sampling (years) ^a	65.0 ± 9.6 (28–92)	45.0 ± 17.0 (21–98)
Male ^a	64.3 ± 10.2 (28–92)	43.6 ± 15.0 (22–92)
Female ^a	65.4 ± 9.9 (28–92)	46.8 ± 19.0 (21–98)
Age at onset (years) ^a	58.0 ± 10.5 (20–88)	
Male ^a	57.7 ± 10.9 (20–88)	
Female ^a	58.3 ± 10.1 (25–82)	

^a Data are mean ± SD (range)

Table 2 Allele frequency of *LRRK2* c. 2264C > T (p. P755L) in Japanese patients with Parkinson's disease and controls of general population

	Genotype, n (%)			Allele, n (%)		χ^2 ^a	OR (95% CI)
	C/C	C/T	T/T	C	T		
Patients (n = 501)	495 (98.8)	6 (1.2)	0 (0)	996 (99.4)	6 (0.6)	0.06	1.15 (0.40–3.32)
Controls of general population (n = 583)	575 (98.6)	8 (1.4)	0 (0)	1,158 (99.3)	8 (0.7)		

^a Compared with the control

OR odds ratio, CI confidence interval

was rare in Asians compared to North Africans or Caucasians (Tomiyama et al. 2006). *LRRK2* variants are reported to spread worldwide with some ethnic differences among each variant, such as R1441G, R1441C, R1441H (exon 31, ROC domain), G2019S, I2020T (exon 41, MAPKKK domain), and G2385R (exon 48, WD40 domain) (Mata et al. 2005). Since *LRRK2* consists of as many as 51 exons, it is important to decide which exon(s) of this gene should be screened first for efficient analysis of mutation in patients with various ethnic backgrounds. In this regard, *LRRK2* exon 41 and 31 are reasonable to be screened first; however, exon 19 is not likely a candidate exon for causative mutation screening in PD. In addition, although MAPKKK and ROC domain are reported to be associated with kinase activity of *LRRK2* (Paisán-Ruíz et al. 2004; Zimprich et al. 2004; Smith et al. 2006), the existence and the role of the predicted ankyrin repeat-like domain in *LRRK2* have not been established yet.

So far, *LRRK2* P755L as well as G2385R variants have been found in only Chinese, Taiwanese, and Japanese (Asians) with similar frequencies in some Asians, but have not been found in Caucasians. Thus, these variants could occur independently in very ancient Asians with a single founder effect (Farrer et al. 2007). Although the HapMap project has been very successful, the presence of ethnic differences among *LRRK2* variants such as G2019S, R1441G, G2385R, and P755L suggest that further establishment of ethnic-specific or native-specific data is essential for more accurate SNP analyses and genome-wide association studies.

Conclusion

Our extended association study in Japanese with large sample size suggests that *LRRK2* P755L is a non-disease-associated polymorphism in PD patients.

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Screening *PARK* genes for mutations in early-onset Parkinson's disease patients from Queensland, Australia

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Abstract

A family history of Parkinson's disease (PD) is the most commonly reported risk factor after age, suggesting a genetic component to the disease in a sub-group of patients. Mutations in at least six genes have been identified that can lead to monogenic forms of PD. We screened a sample of 74 early-onset PD cases out of a cohort of 950 patients (onset <50 years) for genetic abnormalities in known familial Parkinsonism genes. A self-reported family history of PD existed for 30 patients (40.5%). Of these, 13 each had a first- or a second-degree relative with PD and four reported a more distant relative with PD.

The entire coding region of the *PRKN* (MIM 602544), *DJ-1* (MIM 602533) and *PINK1* (MIM 698309) genes, and exon 41 of the *LRRK2* gene (MIM 609007) were screened by direct sequencing. All exons of *PRKN* were examined for gene-dosage abnormalities.

Screening identified five patients with putative genetic disease: two patients carried *PRKN* mutations (p.G12R heterozygous and p.G430D homozygous), one patient carried a p.G411S heterozygous amino acid change in the *PINK1* gene and two individuals were heterozygous for the common p.G2019S mutation in *LRRK2*. No alpha-synuclein or *DJ-1* variants were observed.

Our data suggest that approximately 7% of early-onset PD cases seen in Queensland movement disorders clinics have mutations involving known *PARK* genes.

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Keywords: Parkinson's disease; *PARK* genes; Mutations

1. Introduction

Parkinson's disease (PD) is a neurodegenerative condition with a typical onset in the seventh decade, however, about 4% of PD patients present with early-onset before the age of 50 years [1]. It is a complex, multifactorial disorder, comprising genetic and environmental components. The majority of cases appear to be sporadic or idiopathic, however, in the recent

past a number of mutations in at least six genes (*PARK1*, 2, 5, 6, 7, and 8) have been identified as being causative in the familial form of the condition, accounting for a small number of all PD cases. Mutations in these genes may lead to the disease phenotype and are often characterized by an earlier onset (under the age of 50 years) with or without Lewy body pathology. It is to be expected that more mutations causative for the disease in 'sporadic' PD will be identified in the future, adding to the number of distinct genetic forms of PD. The aetiology of the sporadic form of PD is still unclear but identification of molecular mechanisms and gene products underlying the disease in its monogenic form have shed some light on possible pathways involved in the non-hereditary form of the condition.

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