ORIGINAL CONTRIBUTION

Mutations for Gaucher Disease Confer High Susceptibility to Parkinson Disease

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Background: Increased frequency of pathogenic variants in *GBA*, the causative gene for Gaucher disease, has been suggested to be associated with Parkinson disease (PD).

Objectives: To conduct comprehensive resequencing of *GBA* to identify all sequence variants and to investigate the association of these variants with PD.

Design: Case-control study.

Setting: Multicenter university-based study.

Participants: Five hundred thirty-four patients with PD, 34 families in which multiple patients with PD are present, and 544 control subjects.

Main Outcome Measures: Disease status and *GBA* variations.

Results: Comprehensive resequencing of *GBA* in 534 patients with PD and 544 controls revealed 27 sequence variants: 11 pathogenic variants associated with Gaucher disease, 11 nonsynonymous variants not associated with Gaucher disease, and 5 synonymous variants. Fifty patients with PD (9.4%) had 1 of the 11 pathogenic variants in the heterozygous state, whereas only 2 controls (0.37%) had such variants (odds ratio, 28.0). Among the pathogenic variants, R120W and L444P/Rec*Nci*I were highly prevalent, and each showed a significant association with PD. Furthermore, other rare pathogenic variants were found in 13 patients with PD but not in the controls, further confirming the role of these rare variants in the susceptibility to PD. Patients with PD carrying pathogenic variants were significantly younger than those not carrying them. In addition, concordance of PD states and pathogenic variants was observed in 8 multiplex families with PD.

Conclusion: Heterozygous pathogenic variants in *GBA* confer a high risk for sporadic PD, even for familial clustering, and are associated with significantly earlier age at onset of disease.

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PARKINSON DISEASE (PD), characterized by tremor, rigidity, bradykinesia, and postural instability, is the second most common neurodegenerative disease after Alzheimer disease, with usual onset in late adulthood, that is, after age 50 years. The prevalence

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of PD is estimated to be 0.3% in the general population and 1% in individuals older than 60 years.[1] Although *SNCA*, *LRRK2*, *UCHL-1*, *PARK2*, *PINK1*, and *DJ-1* have been identified as the causative genes for familial PD,[2] patients with PD with pathogenic mutations in these genes are rare. Most cases of PD are sporadic and the etiologies poorly understood. A population-based study coupled with genealogic information demonstrated that the estimated risk ratio for PD in siblings of patients with PD was significantly high ($\lambda s = 6.7$), which suggests that genetic factors substantially contribute to the development of sporadic PD.[3] To elucidate susceptibility genes for sporadic PD, numerous case-control association studies using the analyses of single nucleotide polymorphisms have been conducted under the common disease–common variants hypothesis; however, only a few consistent findings have been observed.[4] Recently, polymorphisms of *SNCA*, a major component of Lewy bodies, a pathologic hallmark of PD, have been reported to be associated with sporadic PD (odds ratio [OR], 1.4-2.0).[5,6]

Several articles have suggested the association of sporadic PD with heterozygous variants in the glucocerebrosidase gene (*GBA*) (OMIM 606463) encoding the enzyme that is deficient in patients with Gaucher disease, an autosomal recessive lysosomal storage disease. Although *GBA*

Table 1. Demographic Data for Study Participants

Variable	Tier 1		Tier 2	
	Patients With PD (n=61)	Control Subjects (n=47)	Patients With PD (n=473)	Control Subjects (n=497)
Age at sampling, mean (SD), y	66.8 (8.2)	58.4 (11.8)	65.2 (9.9)	43.4 (16.4)
Age at onset of PD, mean (SD), y	58.3 (9.9)	NA	58.2 (10.7)	NA
Sex, male to female ratio	1.44	1.76	1.08	1.12

Abbreviations: NA, not applicable; PD, Parkinson disease.

Table 2. Primers for PCR and Sequence Analysis

Primer	Forward	Reverse
PCR		
Exons 1-5	CCTAAAGTTGTCACCCATAC	AGCAGACCTACCCTACAGTTT
Exons 5-7	GACCTCAAATGATATACCTG	AGTTTGGGAGCCAGTCATTT
Exons 8-11	TGTGTGCAAGGTCCAGGATCAG	ACCACCTAGAGGGGAAAGTG
Sequence		
Exon 1	TAGTGGATCCTCTATCCTTC	AAATTCCAGTGCCAGGATTC
Exon 2	AAAGGCAGCTAAGCCCTGCC	GCTACCAAAGGACTATGAGG
Exon 3	AGTCTCTCCTAGCAGATGTG	TCCATGGTGATCACTGACAC
Exon 4	AAATGGTGTCAGTGATCACC	GCAGAGTGAGATTCTGCCTC
Exon 5	GCAAGTGATAAGCAGAGTCC	CAAGCAGACCTACCCTACAG
Exon 6	AATGGCTGAACCGGATGCAC	AAGTGGAACTAGGTTGAGGG
Exon 7	TCAAGTGATCCACCTGCCTC	AGTTTGGGAGCCAGTCATTT
Exon 8	TGTGTGCAAGGTCCAGGATCAG	GCTTCTGTCAGTCTTTGGTG
Exon 9	ACCCTTACCTACACTCTCTG	GTGATGTAAGCCATCCGATG
Exon 10	GGGTGACTTCTTAGATGAGG	AGCTGAGAGTGTGATCCTGC
Exon 11	GGAAGTGGGCTGAAGACAGC	TTAGTCACAGACAGCGTGT

Abbreviation: PCR, polymerase chain reaction.

variants associated with Gaucher disease are diverse and each carrier frequency is rare, most of the previous studies analyzed only specific variants[7-16] and sample sizes were small.[17-21] Therefore, ORs assessed for the *GBA* variants have been highly variable in the subsequent studies, making the medical implications of *GBA* variants associated with PD inconclusive. We conducted extensive resequencing analysis of *GBA* in patients with PD and in control subjects and found that *GBA* variants that are pathogenic for Gaucher disease confer high susceptibility to sporadic PD and, furthermore, familial clustering of PD.

METHODS

SUBJECTS

We conducted a resequencing of *GBA* in patients with PD and control subjects using a microarray-based, high-throughput resequencing system (first tier). As an independent data set, resequencing of *GBA* was conducted on large-scale samples (second tier) using direct nucleotide sequence analysis. The first tier comprised 61 unrelated patients with PD at the University of Tokyo Hospital and 47 controls provided by the Japan Multiple System Atrophy Research Consortium. The second tier comprised 473 unrelated patients with PD and 497 controls provided by the Japanese Parkinson Disease Susceptibility Gene Consortium (**Table 1**). In addition, 34 families in which multiple patients with PD are present (hereafter referred to as "multiplex families") independent of participants in tiers 1 and 2, having more than 1 patient with PD in the second degree, were provided by the Japanese Parkinson Disease Susceptibility Gene Consortium. The diagnosis of PD was based on diagnostic criteria for PD.[22] This study was approved by the institutional review boards of the participating institutions.

GENOMIC DNA AND AMPLIFICATION OF *GBA*

Genomic DNA was extracted from peripheral blood leukocytes using standard procedures. Three primer pairs were designed to selectively amplify *GBA* but not its pseudogene, as previously described (**Table 2**).[23]

RESEQUENCING OF TIER 1

Resequencing of *GBA* was conducted using newly designed resequencing microarrays TKYPD02 and TKYPD03, both of which were composed of tiled sequences of all 11 exons of *GBA* and the flanking 12 base pairs of the splicing junctions.[24] The analysis was conducted according to the manufacturer's instructions (Affymetrix Inc, Santa Clara, California). All variants were further confirmed at direct nucleotide sequence analysis using a genetic analyzer (ABI PRISM 3100; Applied Biosystems Inc, Foster City, California).

RESEQUENCING OF TIER 2

The polymerase chain reaction products were subjected to direct nucleotide sequence analysis for the coding sequences and the flanking splice sites of *GBA* using DNA analyzers (ABI3730xl; Applied Biosystems Inc). The primers for sequence analysis are given in Table 2.

Table 3. Frequencies of the Pathogenic *GBA* Variants in Patients With PD and Control Subjects

Variants	Patients With PD (n=534)	Control Subjects (n=544)	*P* Value[a]	OR (95% CI)
R120W	15	0	<.001	NA
R131C	1	0	NA	NA
N188S	4	0	.06	NA
R120W-N188R-V191G-S196P-F213I	1	0	NA	NA
G193W	1	0	NA	NA
F213I	1	0	NA	NA
R329C	2	0	.25	NA
L444P	8	0	.004	NA
L444P-A456P-V460V (Rec*Nci*I)	14	2	.002	7.3 (1.7-66.4)
A456P-V460V	1	0	NA	NA
R496C	2	0	.25	NA
Total (%)	**50 (9.4)**	**2 (0.37)**	6.9×10^{-14}	28.0 (7.3-238.3)

Abbreviations: CI, confidence interval; NA, not applicable; OR, odds ratio; PD, Parkinson disease.
[a] Fisher exact test.

STATISTICAL ANALYSIS

Standard statistical methods were used to test the difference in carrier frequency (Fisher exact test), to compute ORs and corresponding 95% confidence intervals, and to compare mean age at onset of PD (*t* test). For a meta-analysis, a pooled OR was calculated using a fixed-effects model (Mantel-Haenszel method). $P < .05$ was considered statistically significant. Data were analyzed using commercially available statistical software (StatsDirect version 2.6.5; StatsDirect Ltd, Cheshire, England).

RESULTS

Resequencing of tier 1 (61 patients with PD and 47 controls) revealed that 6 patients with PD carried the variants (1 R120W, 1 R329C, 3 Rec*Nci*I, and 1 R496C) that are pathogenic for Gaucher disease, whereas none of these variants were present in the controls. Given this result, we further expanded the comprehensive resequencing analysis to tier 2 (473 patients with PD and 497 controls) and identified 44 patients with PD carrying the variants that have been reported to be pathogenic for Gaucher disease, whereas these variants were present in only 2 controls.

Pathogenic variants were either single-base substitutions (R120W, R131C, N188S, G193W, F213I, R329C, L444P, and R496C) or complex multiple substitutions (R120W-N188R-V191G-S196P-F213I, L444P-A456P-V460V, and A456P-V460V). The precise structures of the complex alleles were confirmed at nucleotide sequence analysis of the subcloned mutant alleles. Among the complex mutant alleles, L444P-A456P-V460V is a Rec*Nci*I allele, a recombination allele that consists of 3 single-base substitutions of the pseudogene origin in exon 10.[25] In summary, we found that 50 of 534 patients with PD (9.4%) had these pathogenic variants in the heterozygous state, whereas only 2 of 544 controls (0.37%) had such variants in the heterozygous state (OR [95% confidence interval] for patients with PD compared with controls, 28.0 [7.3-238.3], which was highly significant ($P = 6.9 \times 10^{-14}$) (**Table 3**). When individual variants were analyzed, the frequency of the R120W, L444P, and Rec*Nci*I carriers was significantly higher in patients with PD than in controls ($P < .001$, .004, and .002, respectively). In addition, we identified 11 nonsynonymous variants and 5 synonymous variants in tiers 1 and 2, and none of these has been shown to be causative for Gaucher disease. When these variants were analyzed individually and in combination, the frequency of patients with PD was not significantly different from that of the controls (**Table 4**).

We analyzed the clinical manifestations in the 50 patients with PD carrying pathogenic variants in *GBA*. The age at disease onset in the patients with PD who were carriers of such variants was significantly younger than in those who were not carriers (**Table 5**). Detailed clinical data were available for 49 of 50 patients with PD carrying pathogenic variants. Forty-one of 49 patients with PD (83.7%) showed good responsiveness to antiparkinsonian drug treatment. Iodine 123–labeled metaiodobenzylguanidine cardiac scintigraphy[26] was carried out in 33 patients with PD, revealing that 29 of 33 patients with PD (87.9%) had reduced cardiac uptake, consistent with a diagnosis of PD. In the 49 patients with PD, 13 (26.5%) manifested overt dementia (clinical dementia rating[27] ≥1) and 17 (34.7%) developed visual hallucinations during the course of the disease (mean [SD] interval between onset of PD and evaluation of dementia or visual hallucinations, 9.1 [4.1] and 7.9 [5.0] years, respectively). *N*-isopropyl-*p*-[^{123}I]-iodoamphetamine single-photon emission computed tomography was performed in 15 patients with PD, of whom 8 had dementia. All 8 patients with dementia exhibited hypoperfusion in the occipital areas. In the 7 patients without dementia, 5 exhibited hypoperfusion in the occipital areas and 2 had normal findings.

Detailed inquiry into the family history of the 50 patients with PD carrying pathogenic variants in *GBA* revealed that 11 patients (22.0%) had parents or siblings with PD. Genomic DNA was available for 3 affected siblings. All 3 affected siblings had the same *GBA* variants (2 R120W and 1 Rec*Nci*I) as did their probands. Given the concordant *GBA* variants in the 3 affected siblings, we analyzed probands of an additional 34 multiplex families independent of those in tiers 1 and 2 with more than 1 patient (parent or sibling) with PD. We found that 5

Table 4. Frequency of Nonpathogenic *GBA* Variants in Patients With PD and Control Subjects

Variant	Patients With PD (n=534)	Control Subjects (n=544)	*P* Value[a]	OR (95% CI)
I(-20)V	77	66[b]	.28	1.2 (0.84-1.8)
L(-15)F	1	0	NA	NA
L67Q	0	1	NA	NA
V121V	0	1	NA	NA
D153N	1	0	NA	NA
R163Q	4	7	.55	0.58 (0.12-2.3)
P299T	1	0	NA	NA
G307S	1	0	NA	NA
T334I	0	1	NA	NA
L336L	1	0	NA	NA
G344G	1	0	NA	NA
F347L	0	1	NA	NA
R359L	1	0	NA	NA
V460V	2	1	.62	2.0 (0.11-120.7)
K466K	11	8	.50	1.4 (0.51-4.1)
I489V	4	3	.72	1.4 (0.23-9.3)
Nonsynonymous	90	79	.32	1.2 (0.85-1.7)
Synonymous	15	10	.32	1.5 (0.64-3.9)

Abbreviations: CI, confidence interval; NA, not applicable; OR, odds ratio; PD, Parkinson disease.
[a] Fisher exact test.
[b] One subject had I(–20)V in the homozygous state.

Table 5. Age at Onset of PD in Carriers and Noncarriers of the Pathogenic *GBA* Variants

Variable	No. of Patients	Age at Onset of PD, Mean (SD)	*P* Value[a]
Carriers of pathogenic *GBA* variants	50	52.5 (7.4)	<.001
Carriers of R120W	15	51.8 (8.2)	.01
Carriers of L444P/Rec*Nci*I	22	52.6 (6.9)	.007
Noncarriers of pathogenic *GBA* variants	484	58.8 (10.7)	NA

Abbreviations: NA, not applicable; PD, Parkinson disease.
[a] *t* Test.

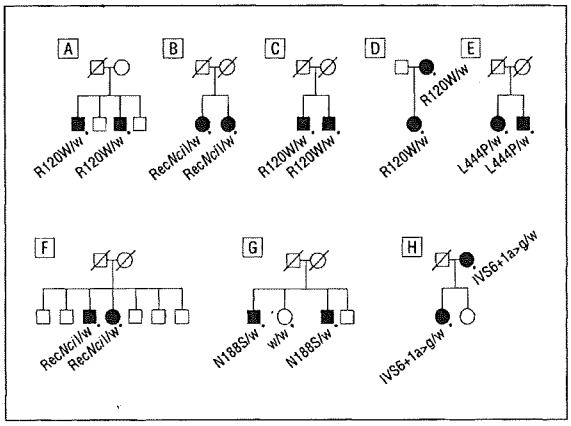

Figure. Pedigree charts for 8 families in which multiple patients with Parkinson disease are present. Squares indicate males; large circles, females; slash, deceased; solid square or large circle, affected individual; and small dot, individual with genomic DNA.

of 34 probands (14.7%) had pathogenic variants in *GBA* (1 each, R120W, N188S, IVS6 + 1g>a, L444P, and Rec*Nci*I), and all 5 affected relatives also concordantly had the same *GBA* variants as did their probands. The splice junction mutation IVS6+1g>a is a novel variant that has not been reported even in patients with Gaucher disease; however, it is likely the pathogenic variant because it would affect splicing of intron 6. In total, 8 multiplex families with patients with PD concordantly carrying the pathogenic variants were identified (**Figure**).

We compared the distributions of pathogenic variants in *GBA* in the 534 Japanese patients with PD (50 alleles) with those of the mutations that have been previously described in the 50 Japanese patients with Gaucher disease (100 alleles).[28] R120W was present in 30% of the pathogenic variants in the patients with PD, whereas it was not described in patients with Gaucher disease. F213I was the second most common mutation in patients with Gaucher disease (14%), but it was present in only 2% of the pathogenic variants in patients with PD. In contrast, the frequency of L444P and Rec*Nci*I was comparable in the 2 groups, and these were the most common variants.

COMMENT

Multiple rare *GBA* variants that are responsible for Gaucher disease confer high risk for PD on the basis of the extensive resequencing of *GBA* of large data sets of Japanese patients with PD and controls. The combined carrier frequency of the pathogenic variants was as high as 9.4% in patients with PD and highly significantly more frequent than in controls (0.37%) with a markedly high OR (95% confidence interval) for patients with PD compared with controls (28.0 [7.3-238.3]). The frequency of nonneuronopathic and neuronopathic Gaucher disease in Japan is estimated to be 1 in 500 000 and 1 in 1 200 000 live births, respectively,[29] which is in accord with the frequency of pathogenic variants in the controls (2 carriers per 544 individuals) in this study.

Among the pathogenic variants identified in the patients with PD, R120W and L444P/Rec*Nci*I were highly prevalent. The identification of multiple rare variants that are pathogenic for Gaucher disease was achieved only by extensive resequencing of large data sets, as clearly demonstrated in the present study. For these pathogenic variants except R120W and L444P/Rec*Nci*I, the frequency of the individual variants was low in patients with PD, and the association with PD should be confirmed in much larger association studies. However, we observed these various rare pathogenic variants in 13 patients with PD, whereas such variants were not observed in the controls. These findings further strengthen the role of these rare *GBA* variants in susceptibility to PD as well.

In contrast to the present findings, previous association studies demonstrated substantially variable ORs. In the studies that demonstrated a significant association of *GBA* variants with PD,[7,11,13-17,20,21] N370S is the variant accounting for most of the significant association. N370S is highly prevalent in the Jewish population, with a carrier frequency of 4% to 6%,[7,8,16,20] and that significant association of N370S with PD has not been demonstrated in other ethnic populations. In contrast to N370S, L444P/Rec*Nci*I has been found regardless of ethnic background. When previous studies that analyzed L444P/Rec*Nci*I[7,9-21] and the present study were subjected to meta-analysis (4181 patients with PD and 9587 controls), a high pooled OR (95% confidence interval) of 6.8 (4.0-11.8) for L444P/Rec*Nci*I was obtained without evidence of significant heterogeneity (Cochran $Q=7.3$; $P=.88$), further confirming the role of *GBA* variants in PD. However, previous studies with small sample sizes failed to detect controls carrying L444P/Rec*Nci*I,[9,11,14,15,17-21] although L444P/Rec*Nci*I was detected in patients with PD. Thus, it is crucially important to determine the frequency of the *GBA* variants in the controls for accurate evaluation of ORs conferred by rare variants, necessitating the analysis of large data sets with at least several hundred patients and controls. Furthermore, there seems to be a bias in the distribution of sequence variants in *GBA* associated with PD compared with that observed in Gaucher disease. In most of the previous studies,[7-16] however, only specific variants considered common in patients with Gaucher disease have been analyzed, which may have led to the underestimation of mutant *GBA* carrier frequency.

Clinically, patients with PD with heterozygous pathogenic variants in *GBA* were significantly younger at disease onset than those without such variants, which confirms findings of previous studies.[7,11,12,16,20] To further determine the exact effects of heterozygous *GBA* variants on PD phenotypes, extensive clinical and epidemiologic analyses should be conducted in large cohorts.

In the present study, we identified 8 multiplex families with patients with PD concordantly having heterozygous pathogenic variants in *GBA*. Given the markedly high ORs caused by heterozygous pathogenic variants in *GBA*, it is conceivable that such variants underlie not only sporadic PD but also familial PD.

The roles of the pathogenic variants in the pathogenesis of PD still needed to be elucidated. Gain of toxic functions of mutant glucocerebrosidase proteins independent of enzyme activities might be involved in the pathogenesis. However, all variants associated with PD are pathogenic variants for Gaucher disease, which raises the possibility that decrease in glucocerebrosidase activities has a role in the pathogenesis of PD. Identification of the splice junction mutation IVS6+1g>a in the present study may further support this notion.

We should emphasize a paradigm shift from the common disease–common variants hypothesis to the common disease–multiple rare variants hypothesis in our search for disease susceptibility genes in sporadic PD, which may be applicable to studies of other diseases. The multiple rare variants can be identified only by extensive resequencing and are difficult to detect in association studies using common single nucleotide polymorphisms. Such multiple rare variants confer strong genetic risks, as demonstrated in the present study, which is also in striking contrast to the low ORs of those identified in genomewide association studies using common single nucleotide polymorphisms. Our results strongly emphasize the importance of conducting a comprehensive resequencing analysis of disease susceptibility genes in detecting even the rarest variants.

In conclusion, we have established *GBA* as a robust and relatively prevalent genetic risk factor for sporadic PD. Further studies of the biological implications of mutant glucocerebrosidase in the pathophysiologic processes of PD are expected to provide new avenues for developing therapeutic measures for PD.

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Levodopa in the early treatment of Parkinson's disease

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Abstract

L-dopa has many advantages as initial therapy for Parkinson's disease (PD). It is safer, more efficacious, associated with fewer adverse effects, few interactions, easier for patients to use and for clinicians to prescribe, and cheaper than dopamine (DA) agonists. Although L-dopa is more likely than DA agonists to introduce motor fluctuations and dyskinesia, L-dopa is also more effective in improving motor function. Furthermore, there is no long-term benefit from delaying L-dopa based on the risk of motor complications or psychiatric symptoms. Many investigations have shown that L-dopa does not accelerate disease progression. Now is the time to re-evaluate L-dopa for initial treatment of PD.

Keywords: Parkinson's disease; L-dopa; DA agonist; Motor fluctuation, Dyskinesia

1. Introduction

Although it is recommended that dopamine (DA) agonists should be chosen as initial treatment for Parkinson's disease (PD), it is time to re-evaluate the use of levodopa for initial treatment of PD.

While many dopaminergic drugs have been introduced, L-dopa therapy has remained the gold standard for symptomatic treatment of PD. L-dopa is safer, more efficacious, associated with fewer adverse effects, has few interactions, is easier for patients to use and for clinicians to prescribe, and it is cheaper than DA agonists. Despite these advantages, many previous guidelines (Fig. 1) [1,2] have stated that for early stage patients with PD it is appropriate to start treatment with a DA agonist unless the patient is either older than 75 years or has dementia. The rationale for these recommendations has been that (1) L-dopa may accelerate disease progression, and (2) DA agonists are less likely to induce motor fluctuation.

2. Is L-dopa really neurotoxic?

Concern that exogenous L-dopa may be neurotoxic and contribute to the progression of PD arises from the oxidative stress hypothesis of PD [3]. Many *in vitro* studies have demonstrated that the addition of L-dopa or DA to cultured dopaminergic neurons increased cell death [4,5]. However, these experiments were performed under non-physiologic conditions; the concentrations of L-dopa and DA in these experiments were high (>5 μM) and exceeded what would be expected in the brains of patients treated with therapeutic doses (<2 μM). Furthermore, some *in vitro* studies have shown that L-dopa and DA are neuroprotective when neurons are co-cultured with glial cells [6,7].

The ELLDOPA study, conducted to show whether L-dopa is toxic for PD patients, was a large, double-blind, randomized, controlled clinical trial comparing three different doses (150, 300, 600 mg per day) of L-dopa with placebo in patients with early PD [8]. At the end of the trial (after a 2-week washout period), the mean Unified Parkinson's Disease Rating Scale (UPDRS) score of patients treated with L-dopa was better than that of the placebo group. The mean UPDRS score of the highest dosage group was the best, even after the washout period. These results suggest that L-dopa is not toxic, and may even be neuroprotective. This study also included evaluation with β-CIT SPECT

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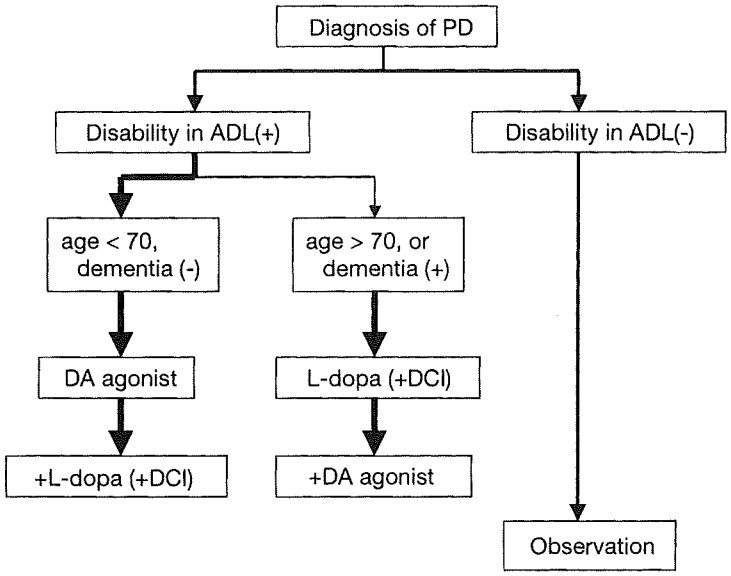

Fig. 1. Guideline for Parkinson's disease (early stage) made by Japanese Society for Neurology, 2002. ADL, activities of daily living; DCI, decarboxylase inhibitor.

imaging, as a marker for intact nigrostriatal dopaminergic neuronal functioning. The imaging studies showed that there was a larger decrease in β-CIT uptake in patients treated with L-dopa in a dose–response manner. These clinical and imaging results suggested that β-CIT SPECT imaging is not appropriate for evaluating the neuroprotective ability of the drugs. Up to that time, several studies had shown that DA agonists are more protective than L-dopa using β-CIT SPECT [9,10] or F-DOPA PET [11]. The results of the ELLDOPA study permitted re-evaluation of the results of these studies, and there is now a consensus that L-dopa does not accelerate disease progression [12].

3. L-dopa is more likely than DA agonists to induce motor fluctuations and dyskinesia

Several clinical studies [9,11] have shown that treatment with DA agonists is less likely to induce motor complications than treatment with L-dopa. Theses studies also showed that L-dopa monotherapy improves activities of daily living and motor function to a greater degree than DA agonists (plus later optional L-dopa). What is of most importance to our patients? The severity of both motor fluctuation and dyskinesia in these studies was low. For example, in the CALM-PD study (4 years) the percentage of disabling dyskinesia was 4.4% in the pramipexole group and 6.9% in the L-dopa group [13]. Furthermore, retrospective investigations failed to show any long-term benefit from delaying L-dopa based on the risk of motor complications, dementia, or psychiatric symptoms [13–16].

Apart from motor complications, the frequency of other common side effects is less with L-dopa than with DA agonists. For example, hallucinations are about three times more likely to occur with ropinirole or pramipexole than with L-dopa [17]. Somnolence, edema, and cardiac valvular fibrosis (pergolide, cabergoline) are also more frequent with DA agonist treatment than with L-dopa [17]. In Japan, those who are taking ropinirole or pramipexole are prohibited from driving because of the risk of sudden onset sleep. Can the incidence of motor fluctuation be reduced only at the expense of improvements in motor function and activities of daily living, and of other side effects such as hallucination, sudden onset sleep, and fibrosis?

4. Mechanism of wearing-off

Disease progression is associated with "wearing off" of therapeutic benefit and the appearance of unpredictable treatment responses, resulting in complex "on-off" response fluctuations. These arise in between doses of L-dopa because the patient no longer has the ability to store dopamine. The other factors driving development of response fluctuations are changes in peripheral L-dopa pharmacokinetics and in post-synaptic function that accompany large-dose and long-term L-dopa therapy [18]. Jenner and colleagues reported on the relation between the amount of lesion and the development of dyskinesia and motor fluctuation by using a 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) marmoset model [19]. In primates with 50% lesions (model of early PD), L-dopa produced an antiparkinsonian response with no induction of dyskinesia. In the group with 75% lesions, L-dopa produced an antiparkinsonian response with a gradual development of dyskinesia. In the >90% lesion group, L-dopa produced a pulsatile anitiparkinsonian response and rapid induction of severe dyskinesia.

Pathological examination in patients with PD demonstrates an exponential loss of nigral pigmented neurons [20]. At 5 years from symptom onset, about 50% of the pigmented neurons remain, compared with age-adjusted controls, and at 10 years 30% remain. The presymptomatic phase of PD, dating from the onset of neuronal loss, was estimated to be about 5 years. Therefore, at the initial symptomatic stage

of the disease there is little possibility of developing motor fluctuation and dyskinesia as long as the appropriate dose of L-dopa is used. In fact, the ELLDOPA study showed the incidence of motor complications in the L-dopa 150 mg and 300 mg groups to be almost equal to that in the placebo group [8].

5. The advantage of L-dopa as initial therapy for Parkinson's disease

The initial use of L-dopa for the symptomatic treatment of PD has many advantages over DA agonists: (1) L-dopa has significantly greater efficacy than DA agonists in alleviating the motor symptoms of PD and improving activities of daily living. (2) Titration of L-dopa to therapeutic levels is much easier and faster than that of DA agonists. (3) L-dopa is much less likely to induce hallucinations, somnolence, edema, or constipation compared to DA agonists. (4) There is a clear cost benefit to using L-dopa. (In Japan, L-dopa + decarboxylase inhibitor 300 mg/day costs about 1 US dollar per day; pramipexole 4.5 mg/day about 16 US dollars; and ropinirole 15 mg/day 26 US dollars.) However, late-developing motor fluctuation and dyskinesia deserve consideration, and the potential for other common side effects such as hallucinations and sleep attacks must also be factored into the treatment decision. Furthermore, early in the course of the disease, L-dopa provides an enduring response that can last several days [21].

There is much evidence to show that DA agonists are efficacious in controlling L-dopa motor fluctuations (as later adjunctive therapy). However, another option is to initiate treatment with levodopa, adding a DA agonist after the first sign of developing motor complications has appeared (early combination).

6. Continuous stimulation by using L-dopa

Continuous daytime intraintestinal infusion of L-dopa can diminish motor complications [22]. This shows that motor complications can be improved by changing the pharmacokinetics of L-dopa. For example, catechol-*O*-methyltransferase (COMT) inhibitors can extend the half-life of serum L-dopa concentration. The duration of L-dopa efficacy can also be extended by taking L-dopa after a meal (Fig. 2). Long-term L-dopa therapy increases the peak L-dopa concentration (C_{max}) and decreases its half-life ($T_{1/2}$) [23]. C_{max} is decreased and $T_{1/2}$ is increased by taking L-dopa after a meal compared to taking it before meals. Therefore, taking L-dopa after meals not only extends effective time but also decreases dyskinesia. By taking L-dopa after meals, the dose of L-dopa can be increased, but the risk of dyskinesia can be decreased. If needed, a low dose of L-dopa may be taken before meals for immediate improvements, with the remainder of the dose taken after meals.

There is much evidence for the efficacy of DA agonists as adjunctive therapy in controlling L-dopa motor fluctuations and dyskinesia [24]. Monoamine oxidase (MAO) B inhibitors [24] and zonisamide [25] may also be used as adjunctive therapy to improve motor fluctuations.

7. Initial symptomatic treatment for early Parkinson's disease

It is appropriate to start treatment of PD with either L-dopa or DA agonists. As highlighted by the American Academy of Neurology practice parameter [17], the choice of initial treatment depends on the relative importance for the patient of improving motor disability and limiting adverse events versus the possibility of lowering the risk of developing long-term motor complications.

The frequency of the development of wearing-off depends on the age of disease onset. Younger-onset patients (younger than 50 years at onset) are more prone to severe dyskinesia and motor fluctuation, while patients older than 70 years at symptom-onset rarely develop disabling dyskinesia and motor fluctuation. Older patients may be more prone to develop hallucinations and other common adverse effects. Therefore, L-dopa is preferred for elderly patients as initial treatment. In younger patients, DA agonists are preferred, but if the patient is at risk of losing his or her job owing to motor disability, L-dopa should be started. Patients whose age of onset is between 50 and 70 years can be prescribed

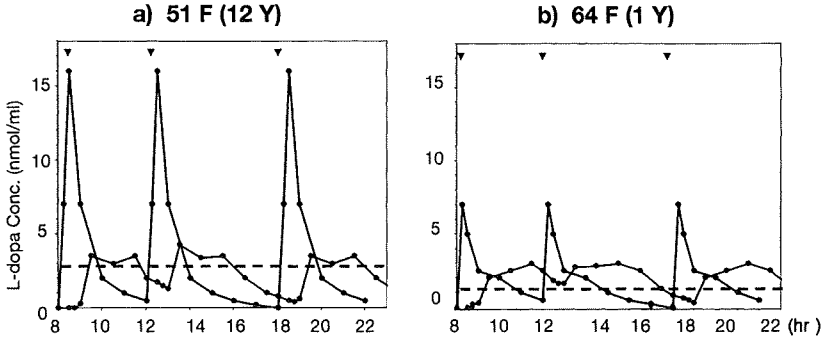

Fig. 2. Effects of a meal on L-dopa pharmacokinetics. (a) 51-year-old woman with 12 years' duration of Parkinson's disease. (b) 64-year-old woman with 1 year duration of Parkinson's disease. A tablet of L-dopa 100 mg + benserazide 20 mg was taken. Black curve: just before meal, red curve: after meal 3 times a day. ▼: meals (at 08.00, 12.00, 18.00 hours). Dashed line: the concentration of effective threshold estimated by clinical symptoms. C_{max} is 2–3 times higher when L-dopa was taken before meals than after meals. L-dopa pharmacokinetics are more stable when taken after meals than before meals.

either L-dopa or a DA agonist. Treatment selection should reflect the patient's needs and under-treatment should be avoided.

Conflict of interest

The author has no conflict of interest to report. No funding applicable.

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ORIGINAL INVESTIGATION

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 FlexGene (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 rs18058754 (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 (Mm)[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	
rs1805874 (*CALB1*)	AC	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	
					MM versus mm	Mm versus mm	M versus m	MM + Mm versus mm	MM versus Mm + mm		
		OR[b] (95% CI)			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)		
rs7684318 (*SNCA*)	CT	Sample Set 1	385/394/89	295/472/165	0.671/0.570	2.7×10^{-9}	5.0×10^{-10}	5.7×10^{-6}	2.8×10^{-8}	0.42/0.31	
		Sample Set 2	213/226/63	323/456/185	0.649/0.572	3.1×10^{-4}	4.5×10^{-5}	0.0013	7.6×10^{-4}	0.80/0.29	
		Combined	598/620/152	618/928/350	0.663/0.571	5.5×10^{-13}	5.1×10^{-14}	8.5×10^{-9}	1.1×10^{-10}	0.65/0.96	
					MM versus mm	Mm versus mm	M versus m	MM + Mm versus mm	MM versus Mm + mm		
		OR[b] (95% CI)			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
		12	Case	Control	Case/Control	Genotype	Allele	11 + 12 versus 22	22 + 12 versus 11	Case/control
Sample Set 1										
rs1805824	*NBN* (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	*DECR1* (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	*DECR1* (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	*DECR1* (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	*CALB1* (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	*CALB1* (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	*CALB1* (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	*DECR1* (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	*CALB1* (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

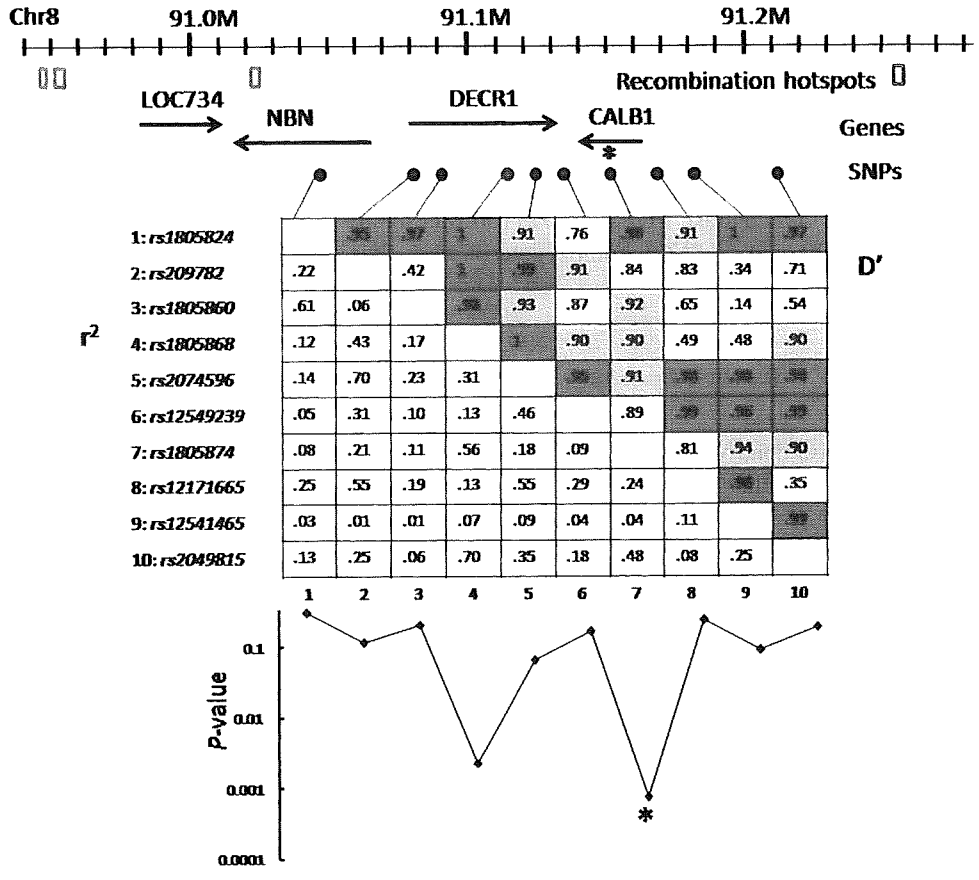

Fig. 1 LD structure and significance of association in the susceptibility region for sporadic PD. *Top* the genomic structure of the *CALB1* region, including genes (*arrows*) and recombination hotspots (*red-lined rectangles*), generated from HapMap. Ten SNPs were plotted (*closed circles*), including 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*
	CALB1 (rs1805874)					
	AA	AC + CC	AA	AC + CC		
SNCA (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
	FGF20 (rs1721100)					
	GG	GC + CC	GG	GC + CC		
SNCA (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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Preventing effects of a novel anti-parkinsonian agent zonisamide on dopamine quinone formation

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Abstract

The neurotoxicity of dopamine (DA) quinones as dopaminergic neuron-specific oxidative stress is considered to play a role in the pathogenesis and/or progression of Parkinson's disease (PD), since DA quinones conjugate with several key PD pathogenic molecules (*e.g.*, tyrosine hydroxylase, α-synuclein and parkin) to form protein-bound quinone (quinoprotein) and consequently inhibit their functions. Zonisamide (ZNS) is used as an anti-epileptic agent but also improved the cardinal symptoms of PD in recent clinical trials in Japan. To evaluate the effects of ZNS on excess cytosolic free DA-induced quinone toxicity, we examined changes in DA quinone-related indices after ZNS treatment both in *in vitro* cell-free system and in cultured cells. Co-incubation of DA and ZNS in a cell-free system caused conversion of DA to stable melanin via formation of DA-semiquinone radicals and DA chrome. Long-term (5 days) treatment with ZNS decreased quinoprotein and increased DA/DOPA chromes in dopaminergic CATH.a cells. ZNS significantly inhibited quinoprotein formation induced by treatment with tetrahydrobiopterin and ketanserin that elevate cytosolic free DA in the cells. Our results suggest that the novel anti-parkinsonian agent ZNS possesses preventing effects against DA quinone formation induced by excess amount of cytosolic DA outside the synaptic vesicles.

Keywords: Zonisamide; Dopamine chrome; Dopamine quinone; Quinoprotein; Parkinson's disease

1. Introduction

Under normal conditions, dopamine (DA) is stable in the synaptic vesicle. However, when levodopa is administered to the damaged dopaminergic neuronal system of Parkinson's disease (PD), a large amount of DA remains in the cytosol outside the synaptic vesicle, since the damaged dopaminergic system has too small DA pool to store DA (Sulzer et al., 2000; Sulzer and Zecca, 2000; Asanuma et al., 2003; Ogawa et al., 2005). The toxicity of excess levodopa and/or DA has been well documented in many *in vitro* and *in vivo* animal studies using parkinsonian models (Ogawa et al., 1993; Basma et al., 1995; Walkinshaw and Waters, 1995; Hastings et al., 1996; Asanuma et al., 2003), despite its marked beneficial effects. Free excess DA is easily metabolized via monoamine oxidase (MAO)-B or by auto-oxidation to produce cytotoxic reactive oxygen species (ROS), and then forms neuromelanin (Sulzer et al., 2000; Sulzer and Zecca, 2000). In the oxidation of DA by MAO, DA is converted to dihydroxyphenylacetic acid (DOPAC) to generate general ROS hydrogen peroxide. On the other hand, non-enzymatic and spontaneous auto-oxidation of DA and L-DOPA produces superoxide and reactive quinones such as DA quinones and DOPA quinones (Tse et al., 1976; Graham, 1978). DA quinones are also generated in the enzymatic oxidation of DA by prostaglandin H synthase (cyclooxygenase-2), lipoxygenase, tyrosinase and xanthine oxidase (Korytowski et al., 1987; Rosei et al., 1994; Hastings, 1995; Foppoli et al., 1997; Chae et al., 2007). These quinones are oxidized to the cyclized aminochromes: DA chrome (aminochrome) and DOPA chrome, and then are finally polymerized to form melanin. Although ROS generation by the auto-oxidation of DA may explain widespread

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toxicity but not specific damage of DA neurons, the highly reactive DA quinone or DOPA quinone itself exerts predominant cytotoxicity in DA neurons and surrounding neural cells, since these quinones are generated from free cytosolic DA outside the synaptic vesicle or from L-DOPA (Sulzer et al., 2000).

The generated DA quinones covalently conjugate with the sulfhydryl group of cysteine on functional proteins, resulting predominantly in the formation of 5-cysteinyl-DA (Graham, 1978; Fornstedt et al., 1986). DA quinones conjugate with cysteine residues of various functional proteins including several key molecules involved in the pathogenesis of PD (*e.g.*, tyrosine hydroxylase, DA transporter and parkin) to form protein-bound quinones (quinoproteins), and inhibit the function of these proteins to cause DA neuron-specific cytotoxicity (Xu et al., 1998; Kuhn et al., 1999; Whitehead et al., 2001; LaVoie et al., 2005; Machida et al., 2005). We reported previously that repeated levodopa administration elevated striatal DA turnover and formation of quinoproteins specifically in the parkinsonian side, but not in the control side, of hemi-parkinsonian models (Ogawa et al., 2000; Asanuma et al., 2005; Miyazaki et al., 2005). Therefore, the excess amount of cytosolic DA outside the synaptic vesicles after levodopa treatment may exert neurodegenerative effects through quinone generation, at least in the damaged dopaminergic nerve terminals. The DA-induced formation of DA quinones and the consequent dopaminergic cell damage *in vitro* and *in vivo* could be prevented by treatment with superoxide dismutase, glutathione, and certain thiol reagents through their quinone-quenching activities (Offen et al., 1996; Lai and Yu, 1997; Kuhn et al., 1999; Haque et al., 2003). We also demonstrated recently that DA agonists pergolide and pramipexole exhibit quenching properties against *in vitro* generated DA-semiquinone radicals (Asanuma et al., 2005; Miyazaki et al., 2005), and that pergolide effectively prevented repeated levodopa-induced elevation of striatal quinoprotein specifically in parkinsonian models (Miyazaki et al., 2005). Thus, DA quinones act as neurotoxic compounds by eliciting dopaminergic neuron-specific oxidative stress and thus play a role in the pathogenesis and/or progression of PD and neurotoxin-induced parkinsonism (Choi et al., 2003, 2005; Asanuma et al., 2004; LaVoie et al., 2005; Machida et al., 2005; Ogawa et al., 2005; Chae et al., 2007).

Zonisamide (1,2-benzisoxazole-3-methaesulfonamide: ZNS), which was originally synthesized in Japan, has been used as an anti-epileptic agent in Japan, South Korea, USA and Europe. An open trial of ZNS (50–200 mg/day) administered in conjunction with anti-PD drugs showed lessening of symptoms, especially wearing-off (Murata et al., 2001), and induced more than 30% improvement of UPDRS total score up to 3 years (Murata, 2004). The addition of ZNS to levodopa treatment of patients experiencing 'wearing-off' fluctuations resulted in lessening of motor fluctuation and significant improvement of the duration, severity and activities of daily living in 'off' time and score of motor examination. Furthermore, a recent nationwide double-blind controlled study in Japan reported that an adjunctive treatment with lower dose of ZNS (25–100 mg/day) to levodopa improved all the cardinal symptoms of PD (Murata, 2004; Murata et al., 2007).

Several pharmacological effects of ZNS have been proposed to be related to its beneficial effects on PD. ZNS is a specific T-type Ca^{++} channel blocker (Suzuki et al., 1992; Kito et al., 1996), which increases burst firing of dopaminergic neurons in the substantia nigra. A single dose of ZNS increased intracellular and extracellular DOPA, DA and homovanillic acid (HVA) levels and decreased DOPAC level in the rat striatum presumably through its moderate MAO-inhibiting effect (Okada et al., 1992, 1995). Long-term administration of ZNS increased activity and protein level of tyrosine hydroxylase in the rat striatum (Murata, 2004), and thus enhanced DA synthesis. However, these effects cannot fully explain the mechanism of its therapeutic effects on levodopa-induced adverse effects.

To evaluate the effects of ZNS on excess cytosolic free DA-induced quinone toxicity, we examined changes in DA quinone-related indices after ZNS treatment both in *in vitro* cell-free DA-semiquinone generating system and in cultured dopaminergic neuronal cells.

2. Materials and methods

2.1. Materials

DA hydrochloride and L-DOPA were purchased from Wako Pure Chemical (Tokyo, Japan) and Sigma (St. Louis, MO), respectively. ZNS and its sodium salt were provided by Dainippon Sumitomo Pharma (Osaka, Japan).

2.2. ESR spectrometry of generated DA-semiquinone radicals

The spectra of semiquinone radicals generated from DA in a cell-free system were recorded with an electron spin resonance (ESR) spectrometer (JES-FR30, JEOL Co., Tokyo) using a flat quartz cuvette as reported previously (Korytowski et al., 1987; Haque et al., 2003). DA or L-DOPA was dissolved in 10 mM sodium phosphate buffer (PB: pH 7.4), and the pH was adjusted to 7.0 by adding 0.1 M NaOH at 4 °C. For the experiment on time-dependency, the pH-adjusted DA or L-DOPA (final concentration 1 mM) was immediately incubated with ZNS sodium salt dissolved in 10 mM PB (final concentration 8 mM, pH 10.8) for 1–60 min at 37 °C, and the spectra for these combinations were analyzed. As a positive control to generate DA-semiquinone, tyrosinase (final concentration 12.5 μg/ml) was incubated instead of ZNS. Furthermore, 0.1 N NaOH (pH 10.9) or pH-adjusted 10 mM PB (pH 10.8) was used instead of ZNS as a negative control. For the experiment on dose-dependency, the pH-adjusted DA (final concentration 1 mM, pH 7.0), with or without various concentrations (ranging from 2 to 8 mM) of ZNS sodium salt dissolved in 10 mM PB (pH 10.8), was immediately incubated for 1 min at 37 °C, and the spectra for these combinations were analyzed. The pH of each final incubation mixture was approximately 8.0. The signal intensity was evaluated by the relative peak height of the second signal of the semiquinone radical spin adduct (the peak height of the second signal is higher than the others and is directly proportional to double integration of the spectra) to the intensity of the Mn^{2+} signal, which was used as the internal standard to correct for measurement error. The conditions of the ESR spectrometer to estimate the semiquinone radical, including magnetic field, power, modulation frequency, modulation amplitude, response time, temperature, amplitude, and sweep time were 335 ± 5 mT, 4 mW, 9.41 GHz, 79 μT, 0.1 s, 25 °C, 1 × 1000 and 1 min, respectively. Furthermore, the levels of DA and its metabolites in the reaction mixture were measured by high-performance liquid chromatography (HPLC) as described previously (Ogawa et al., 2000; Asanuma et al., 2005).

2.3. Effects of ZNS on generation of DA chrome

To examine the effects of ZNS on generation of DA chrome in a cell-free system, pH-adjusted 1 mM DA in 10 mM PB (pH 6.8) and 0.2% Triton X-100 solution were incubated with or without 200 μM ZNS dissolved in 10 mM PB (pH 6.8) for 1 min to 3 h at 37 °C. The level of DA chrome in the final mixture (pH 6.8) was estimated by measuring absorbance of incubation mixture at 475 nm.

2.4. Cell culture and drug treatment

Dopaminergic CATH.a cells (ATCC; #CRL-11179), derived from mouse DA-containing neurons, were cultured at 37 °C in 5% CO_2 in RPMI 1640 culture medium (Invitrogen, San Diego, CA) supplemented with 4% fetal bovine serum, 8% horse serum, 100 U/ml penicillin and 100 μg/ml streptomycin. Cells were seeded in 6-well plates (Becton Dickinson Labware, Franklin Lakes, NJ) for the extraction of cell lysates used for the measurement of protein-bound quinone and DA/DOPA chrome at a density of 1.0×10^5 cells/cm^2. After 24 h, CATH.a cells were exposed to 1–100 μM ZNS diluted in phosphate buffered saline (PBS) for 5 days for the measurements of quinoprotein and DA/DOPA chrome. To examine the effects of ZNS on excess cytosolic free DA-induced quinone elevation, CATH.a cells were exposed simultaneously to 1–100 μM ZNS with 100 μM tetrahydrobiopterin (BH_4) and 10 μM ketanserin, which enhance DA synthesis and blocks vesicle monoamine transporter, respectively (Choi et al., 2005), for 3 h before extraction of total cell lysates for quinoprotein measurement.

2.5. Protein-bound quinone; quinoprotein measurement

Total cell lysates from drug-treated CATH.a cells were prepared with 10 μg/ml phenylmethylsulfonyl fluoride (Sigma) in ice cold-RIPA buffer [PBS (pH 7.4), 1% nonidet P-40 (NP-40), 0.5% sodium deoxycholate and 0.1% sodium dodecyl sulfate]. The nitrobluetetrazolium (NBT)/glycinate colorimetric assay was performed to detect protein-bound quinones (quinoprotein) (Paz et al., 1991). The cell lysate was added to 500 μl of NBT reagent (0.24 mM NBT in 2 M potassium glycinate, pH 10.0) followed by incubation in the dark for 2 h under constant shaking. The absorbance of blue-purple color developed in the reaction mixture was measured at 530 nm.

2.6. Measurement of DA/DOPA chrome in CATH.a cells

For the measurement of DA/DOPA chrome, cells were solubilized in 500 μl of 1% Triton X-100 solution for 2 h and then centrifuged at 20,000 × *g* for 30 min at 4 °C. The supernatant was used as cell extract and incubated for 3 min at room temperature. The level of DA/DOPA chrome was calculated by measuring absorbance of incubated cell extract at 475 nm. Absorbance values in the *in vitro* incubation of DA (0–500 μM) with tyrosinase (10 μg/ml) for 30 min were used as a standard to calculate the concentration of DA/DOPA chrome in the cell extract.

2.7. Protein measurement

The protein concentration was determined using the Bio-Rad protein assay kit or Bio-Rad DC protein assay kit (Bio-Rad, Richmond, CA), based on the method of Bradford and Lowry, respectively, using bovine serum albumin as a standard.

2.8. Statistical analysis

Results are expressed as mean ± S.E.M. values. Statistical analysis of the data was performed using one-way ANOVA followed by *post hoc* Fisher's PLSD test. A *p*-value less than 0.05 denoted the presence of a statistically significant difference.

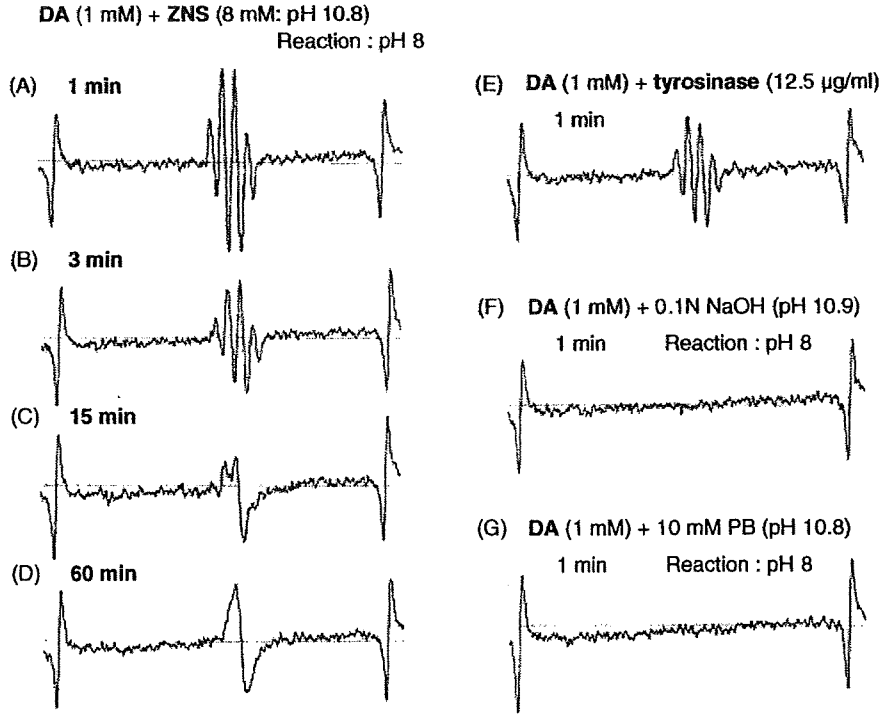

Fig. 1. Effects of ZNS on DA-semiquinone radicals generated from DA in a cell-free system. (A–D) Representative time-course changes in ESR spectra of DA-semiquinone radicals (A–B) to melanin (D) in the incubation of pH-adjusted 1 mM DA (pH 7.0) with 8 mM ZNS (sodium salt) in 10 mM PB (pH 10.8) for 1–60 min at 37 °C at pH 8.0 (incubation mixture). (E) Formation of DA-semiquinone radicals via tyrosinase (12.5 μg/ml)-catalyzed oxidation of 1 mM DA. (F and G) No signals for radical formation at pH 8.0 (incubation mixture) when pH-adjusted 1 mM DA (pH 7.0) was incubated for 1 min at 37 °C with 0.1 N NaOH (pH 10.9) (F) or pH-adjusted 10 mM PB (pH 10.8) (G). Each experiment was performed as triplicate assays.

3. Results

3.1. Effects of ZNS on generated DA-semiquinone radicals in a cell-free system

When a high dose of DA (5 mM) was incubated at 37 °C at neutral pH 7–8, the formation of DA-semiquinone radicals started immediately within 1 min, peaked at around 1 min, then gradually decreased and continued for 10 min, as shown in a previous study (Haque et al., 2003). In the present ESR study, however, no signals for radical formation were detected at pH 8 (incubation mixture) when a lower dose of pH-adjusted 1 mM DA (pH 7.0) was incubated for 1 min at 37 °C with 0.1 N NaOH (pH 10.9) or pH-adjusted 10 mM PB (pH 10.8) (Fig. 1F and G). Interestingly, when pH-adjusted 1 mM DA (pH 7.0) was incubated at 37 °C with 8 mM ZNS (sodium salt) in 10 mM PB (pH 10.8), the formation of DA-semiquinone radicals, which was identified by four waves in ESR spectrometry, started immediately within 1 min and peaked at around 1 min, at pH 8 (incubation mixture) (Fig. 1A and B), as well as formation of DA-semiquinone radicals via tyrosinase-catalyzed oxidation of DA (Fig. 1E). Then, the DA-semiquinone radical induced by incubation of DA and ZNS converted to melanin, which was recognized by a wide single wave, at 15–60 min (Fig. 1C and D). The incubation of pH-adjusted 1 mM DA (pH 7.0) and 2–8 mM ZNS (sodium salt, pH 10.8) at pH 8 (incubation mixture) resulted in DA-semiquinone radical formation at 1 min (Fig. 2A–D) and subsequent melanin formation at 60 min (data not shown) in a ZNS concentration-dependent manner. Furthermore, incubation of pH-adjusted 1 mM L-DOPA (pH 7.0) with 8 mM ZNS (sodium salt, pH 10.8) at 37 °C at pH 8 (incubation mixture) resulted in immediate generation of DOPA-semiquinone radicals within 1 min, with a peak at around 1 min, and conversion to melanin within up to 20 min (Fig. 2E–G). DOPAC, 3-methoxy tyramine and HVA, which are metabolites of DA via MAO and/or catecholamine *o*-methyltransferase, were not detected in any incubation mixture of pH-adjusted DA and ZNS (sodium salt) at either dose of ZNS or incubation time by HPLC (data not shown).

3.2. Effects of ZNS on generation of DA chrome in a cell-free system

Because a high dose of ZNS was required to detect the conversion effects from DA to melanin in ESR spectrometry, we used sodium salt of ZNS, which is highly soluble in 10 mM PB, at a dose of 2–8 mM. However, the pH value of the mixture of ESR examination was slightly alkaline at 8.0 because of high alkalinity of ZNS sodium salt solution (pH 10.8) in the cell-free system. To examine the effects of relatively low dose of ZNS on conversion of DA to melanin at neutral pH, we evaluated generation of DA chrome, which is an intermediate in conversion of DA quinone to melanin, using 200 μM ZNS at pH 6.8, but not its sodium salt (Fig. 3). Although the incubation of pH-adjusted 1 mM DA (pH 6.8) alone at 37 °C showed time-dependent but not significant increases in DA

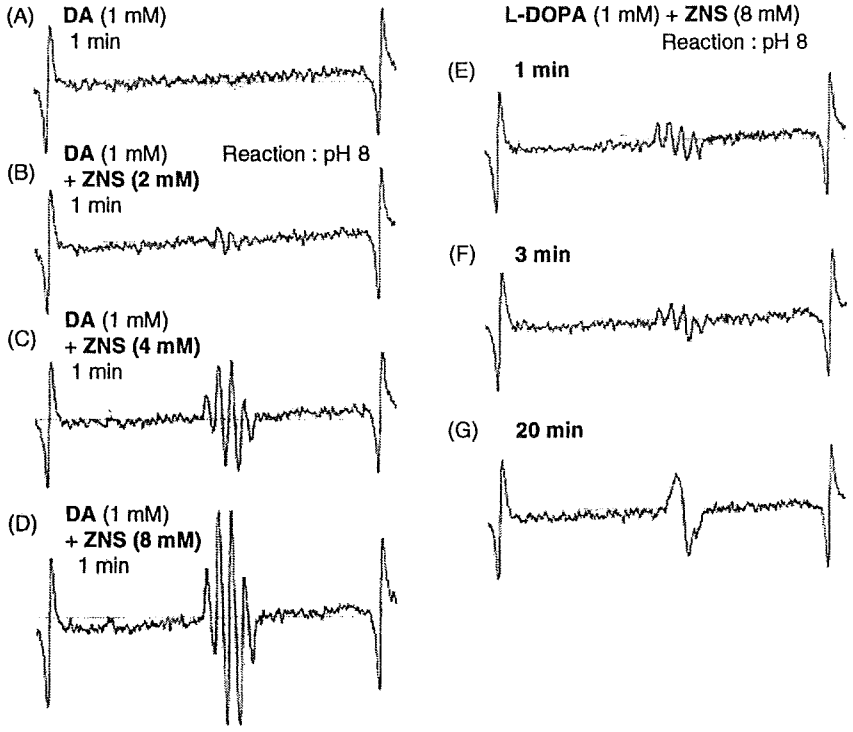

Fig. 2. Effects of ZNS on semiquinone radicals generated from DA or L-DOPA. (A–D) Dose-dependent effects of ZNS on DA-semiquinone radicals generated from DA in a cell-free system. The pH-adjusted 1 mM DA (pH 7.0) was simultaneously incubated with 2–8 mM ZNS (pH 10.8) at pH 8.0 (incubation mixture) for 1 min at 37 °C, and then the relative signal intensity of DA-semiquinone radicals was measured by ESR spectrometry. (E–G) Representative time-course changes in ESR spectra of DOPA-semiquinone radicals (E and F) to melanin (G) in the incubation of pH-adjusted L-DOPA (1 mM, pH 7.0) with 8 mM ZNS (sodium salt) in 10 mM PB (pH 10.8) for 1–20 min at 37 °C at pH 8.0 (incubation mixture). Three independent assays were performed in each experiment.