Table 2
Comparison of the mean PWV and the PAI between cases with and without atherosclerosis-related diseases

Atherosclerosis-related diseases	n^a (%)	Disease	No disease	P^b
Pulse wave velocity (m/s)				
Hypertension	198 (65%)	9.89 (±1.75)	9.00 (±1.61)	<0.001
Diabetes mellitus	52 (17%)	9.80 (±1.84)	9.53 (±1.73)	n.s.
Hyperlipidemia	71 (23%)	9.57 (±1.58)	9.59 (±1.81)	n.s.
Ischemic heart disease	78 (26%)	9.71 (±1.77)	9.54 (±1.75)	n.s.
Cerebrovascular disease	109 (36%)	9.74 (±1.85)	9.49 (±1.70)	n.s.
Pathological atherosclerotic index (points)				
Hypertension	180 (65%)	4.78 (±1.42)	3.93 (±1.26)	<0.001
Diabetes mellitus	49 (18%)	5.18 (±1.32)	4.34 (±1.40)	<0.001
Hyperlipidemia	66 (24%)	4.71 (±1.57)	4.42 (±1.37)	n.s.
Ischemic heart disease	70 (25%)	5.17 (±1.39)	4.26 (±1.36)	<0.001
Cerebrovascular disease	98 (36%)	4.75 (±1.49)	4.35 (±1.37)	<0.05

Values are mean (±S.D.) of the PWV and PAI. PWV indicates pulse wave velocity; PAI, pathological atherosclerotic index; and n.s., not significant.
[a] Number of cases with an atherosclerosis-related disease, with percentages indicated in parentheses.
[b] Student's *t*-test.

A multiple regression analysis of the PWV in all the examinations ($n=3454$) showed significant contributions of systolic blood pressure ($\beta=0.448$, $P<0.0001$) and age ($\beta=0.183$, $P<0.0001$).

Underlying diseases and complications of atherosclerosis were frequently encountered, as shown in Table 2. The mean PWV was significantly different between hypertensive and normotensive cases. No significant differences of the PWV were observed in relation to other diseases.

3.3. Vascular pathology

The average atherosclerotic scores varied significantly among the individual arteries ($P<0.001$), as shown in Table 3. The scores for the aorta and arteries of the lower extremities (common iliac and femoral arteries) were high, while those for the visceral arteries (splenic and superior mesenteric arteries) were low. A majority (85%) of the cases showed moderate to severe aortic atherosclerosis. There were no significant differences in the scores between the left-sided and right-sided arteries in the case of arteries examined bilaterally (common carotid, subclavian, common iliac and external iliac arteries).

The histogram of the PAI revealed a normal distribution with a mean (±S.D.) of 4.5 (±1.4) points and a range from 1.1 to 7.9 points.

3.4. Clinical data and vascular pathology

There was a positive correlation between the age at death and the PAI ($n=275$, $\gamma=0.219$, $P<0.001$). There was no significant difference in the PAI between the two sexes ($P>0.5$). There were significantly positive correlations between the mean systolic pressure and the PAI ($\gamma=0.175$, $P<0.005$), and between the mean pulse pressure and the PAI ($\gamma=0.191$, $P<0.005$); on the other hand, there was no significant correlation between the mean diastolic pressure and the PAI ($\gamma=0.002$, $P>0.5$).

As shown in Table 2, significant differences of the mean PAI were found between cases with and without atherosclerosis-related diseases, except for the case of hyperlipidemia. Table 2 indicates that the PAI is

Table 3
Atherosclerotic score of individual arteries and its correlation with the mean PWV

Name of arteries (a.)	*n*	Atherosclerotic score	Correlation with mean PWV	*P*
Common carotid a.	301	3 (2–4)	0.286	<0.001
Subclavian a.	301	4 (2–5)	0.318	<0.001
Aorta	299	7 (6–8)	0.239	<0.005
Splenic a.	297	2 (0–5)	0.300	<0.001
Superior mesenteric a.	290	2 (0–4)	0.334	<0.001
Common iliac a.	301	8 (6–8)	0.291	<0.005
External iliac a.	301	4 (2–6)	0.248	<0.005
Left femoral a.	300	6 (4–8)	0.330	<0.001
CSI	304	7 (10–12)	0.186	<0.005
BAI	268	3.2 (1.8–4.4)	0.208	<0.001

Values in each column are number of cases examined, the median of the atherosclerotic scores with percentiles (25–75%) indicated in parentheses, Spearman's correlation coefficients (ρ) between the atherosclerotic score and the mean PWV, and the level of significance. PWV indicates pulse wave velocity; CSI, coronary stenotic index (range: 0–15); BAI, brain atherosclerotic index (range: 0–8).

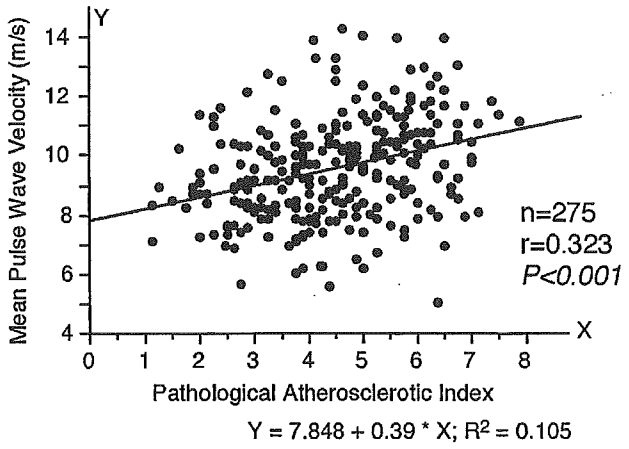

Fig. 2. Correlation between the pathological atherosclerotic index and the mean pulse wave velocity.

more related to atherosclerosis-related diseases than the PWV.

3.5. Vascular pathology and PWV

As shown in Table 3, there were positive correlations between the atherosclerotic scores of the individual arteries and the mean PWV. When the arteries with atherosclerosis more than moderate degree (≥6 points) were regarded as the atherosclerotic arteries, a significant correlation was present between the total numbers of the atherosclerotic arteries and the PWV ($\gamma = 0.335$, $P < 0.0001$). There was also a significantly correlation between the PAI and the mean PWV ($\gamma = 0.323$, $P < 0.001$), as shown in Fig. 2. Table 4 summarizes the correlations between the PAI and the mean PWV under specific conditions. In normotensive cases, a positive correlation was observed ($\rho = 0.336$, $P < 0.005$) between the PAI and the mean PWV. The Spearman's correlation coefficient was higher in the cases that underwent repeated PWV examinations ($\rho = 0.390$) than in those who underwent a single examination ($\rho = 0.312$). The correlation between the PAI and the mean PWV was studied with respect to the premortem intervals between the date of the last examination and the date of death. The Spearman's correlation coefficient between the PAI and the mean PWV was low ($\rho = 0.284$) in cases with an intermediate interval (2–4.9 years), and high ($\rho = 0.459$) in those with a long interval (≥5 years), while it was intermediate ($\rho = 0.337$) in the cases where the interval was short (<2 years). Thus, the correlation between the PAI and PWV seemed to be independent of the premortem intervals in the present autopsy cases.

Table 4
Correlations between the PAI and mean PWV

	n	Correlation coefficients (ρ)	P
All autopsy cases	275	0.353	<0.001
Blood pressure			
Normotensive	95	0.336	<0.005
Hypertensive	180	0.271	<0.001
Number of PWV examinations			
Single	124	0.312	<0.001
Multiple	151	0.390	<0.001
Premortem interval[a]			
0–1.9 years	87	0.337	<0.005
2.0–4.9	89	0.284	<0.01
≥5.0	99	0.459	<0.001

Values are Spearman's correlation coefficients (ρ) between the PAI and PWV. PAI indicates pathological atherosclerotic index; PWV, pulse wave velocity.
[a] Premortem interval between the date of the last PWV examination and the date of death.

Table 5
Multiple regression analysis of the pulse wave velocity in autopsy cases ($n = 304$)

	Standardized partial regression coefficients (β-values)	Correlation coefficients (γ-values)
Mean systolic blood pressure	0.444*	0.038
Age at death	0.154**	0.036
PAI	0.209*	0.258
Coefficient of determination (R^2)	0.331	

PAI: pathological atherosclerotic index.
* $P < 0.0001$.
** $P < 0.005$.

A "changing rate" of PWV was defined as a regression coefficient of PWV in simple regression analysis. The meta-regression analysis showed a positive correlation between the changing rate of PWV and the PAI, independent of the mean PWV values. Each 1 m/s increase of PWV per month corresponded to an increase of PAI of 0.69 points.

A multiple regression analysis was performed to determine the contributions of the major decisive factors of the PWV. Three independent factors, namely, age at death, mean systolic blood pressure and the PAI, were selected for the analysis. The result of the analysis was shown in Table 5. The standardized partial regression coefficient and correlation coefficient between the PAI and the mean PWV were 0.209 and 0.258, respectively. Thus, the PAI accounted for only 5% (=0.209 × 0.258) of the variability of the variance of the PWV.

4. Discussion

Arteriosclerosis comprises three different pathological entities: atherosclerosis of elastic or muscular arteries, Monckeberg arteriosclerosis (medial calcific sclerosis) of muscular arteries, and arteriolosclerosis. All of these entities are very common in the elderly and, in the present study, 85% of the subjects showed moderate to severe atherosclerosis of the aorta. In the early stage of atherosclerosis, the intima is involved, but the subsequent medial destruction and fibrosis

result in arterial dilatation and decrease of elasticity. Meanwhile, the PWV reflects the mechanical properties of the arterial wall, especially the amount and structure of the elastic fibers in the media, and is closely correlated with the blood pressure. The present study revealed a weak, but positive correlation between the PAI and the PWV in normotensive subjects. A changing rate of the PWV, as well as the PWV itself, was correlated with the PAI. In other words, a higher changing rate meant more severe atherosclerosis. Takahashi performed serial PWV measurements in a variety of patients and the results were consistent with ours, in that the changing rate of the PWV was especially high in cases of hypertension, ischemic heart disease and diabetes mellitus [17]. The correlation coefficient between the PAI and the PWV was higher in those cases who underwent repeated examinations than in the cases who underwent a single examination. These results led us to conclude that repeated PWV measurements could give us precise information about the severity of atherosclerosis.

The blood pressure and age of the patients are both important factors influencing the PWV. But, it is uncertain whether the high PWV values among hypertensive patients and the elderly are caused by the physiological responses to high blood pressure and the aging process, or by the concomitant atherosclerosis which is also common in these subpopulations. Multiple regression analysis in the present study showed that atherosclerosis was a more important factor influencing the PWV than the age of the patients. Earlier reports stated that aging is the dominant factor responsible for reduced arterial compliance, in other words, increases of PWV, and that the concomitant atherosclerosis is an additional factor of arterial stiffness [1,2]. The decrease and fragmentation of elastic fibers and increase of collagen fibers in the arterial wall associated with aging are considered to be the cause of the increase of PWV. Hosoda et al. reported that the elastic fiber contents of the aorta in non-atherosclerotic cases sharply decreased from 40% in neonates to 27% in the sixth decade, but it remained unchanged thereafter until the ninth decade [18]. Since the cases examined in the present study were all elderly people over the age of 67 years, Hosoda's data were not inconsistent with our results.

To the best of our knowledge, there is no report so far in English medical literature of the existence of a correlation between the PWV and the severity of atherosclerosis as examined by pathological methods. Otuska [19] performed a pathological study on this subject based on 72 autopsy examinations and reported, in a Japanese journal, the existence of a positive correlation between the PWV and pathological changes in the aorta, especially the intimal changes seen in atherosclerosis. Farrar et al. [20,21], in a series of experimental studies on non-human primates, reported that atherosclerosis caused thickening of the arterial wall and medial degeneration, which caused an increase of the PWV. He also observed that discontinuation of a diet high in fat caused regression of atheroma and decrease of the PWV values [22].

Blacher et al. [10] studied the correlations between the PWV and the severity of atherosclerosis in selected arteries in hypertensive patients. In their studies, the diagnosis of the atherosclerosis depended on clinical information and imaging techniques such as the carotid echography and abdominal CT. Our study was different from the other studies including the one by Blacher et al., in that the assessment was based on the pathological examination, which can detect the subclinical or uncomplicated atherosclerosis. They reported a good correlation between the PWV and the severity of atherosclerosis in aorta and the arteries of the lower limbs. The present study showed a relatively good correlation with the PWV in the femoral artery and a relatively weak one in the aorta.

Some limitations of our study warrant attention. As the intervals between the date of the PWV measurements and the date of death were occasionally very long, in some cases more than 5 years, the atherosclerosis might have progressed or regressed after the last examination. In the present study, however, a long interval was not associated with any decrease in the correlation coefficient between the PAI and the PWV.

In several reports, high PWV were described in the patients with hypertension, diabetes mellitus, hyperlipidemia, ischemic heart disease and cerebrovascular diseases. In contrast, the differences in the PWV were statistically significant only in hypertension in the present study. Furthermore, the correlation between PAI and PWV seems so weak, considering only 5% of the variability of PWV was explained by PAI. We frequently observed that the pathological features differ significantly from case to case among the cases with the same degree of atherosclerosis. These features included presumable important influencing factors of the PWV, such as the degree of medial destruction, the diameter of the arterial lumen, and the presence of calcification. This pathological variety may result in a low correlation between the severity of atherosclerosis and the PWV. The weak correlation might be caused by the long premortem intervals mentioned above. Since the tonometric assessment employed in this study is known to less accurate and reproducible than a newly developed oscillometric method [23]. Future autopsy study with an oscillometric PWV measurement is expected to reveal a better association between atherosclerosis and PWV.

5. Perspectives

The PWV examination has become a powerful screening tool to detect the subjects with high cardiovascular and cerebrovascular risks. The present study provides indisputable pathological evidence that the PWV correlates with the severity of aortic and systemic atherosclerosis. Meanwhile, a better association of the PAI to atherosclerosis-related diseases than that of the PWV indicated that the pathological assessment (PAI) of atherosclerosis was superior to its physiological assessment (PWV). Since pathological examinations of atherosclerosis are impossible to perform in living subjects, the development of alternative noninvasive imaging tech-

niques for morphological assessment, such as CT or MRI, is necessary and would be of great benefit in the future.

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European Urology

European Urology 48 (2005) 752–759

The Val158Met Polymorphism of the Catechol-*O*-methyltransferase Gene is Associated with the PSA-Progression-Free Survival in Prostate Cancer Patients Treated with Estramustine Phosphate

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Received 16 May 2005; accepted 14 July 2005

Available online 1 August 2005

Abstract

Objective: The aim of our study is to find out the good responders for estramustine phosphate (EMP) therapy in patients with prostate cancer. We have focused on the metabolism of EMP and studied the association between a functional single-nucleotide polymorphism in the catechol-*O*-methyltransferase gene (Val158Met of COMT) and PSA-progression-free survival in Japanese patients with prostate cancer treated by EMP.

Methods: Seventy-two Japanese patients with previously untreated prostate cancer who were found to be eligible for low-dose EMP therapy were enrolled in the study. Genotyping of the Val158Met polymorphism of COMT was conducted by both the polymerase chain reaction-based restriction fragment length polymorphism method and TaqMan assay.

Results: Patients with the Val/Val genotype of COMT had a significantly higher PSA-progression-free rate as compared to those with the Val/Met or Met/Met genotype ($p = 0.027$). The adjusted hazard ratio of biochemical PSA failure for the Val158Met genotype of COMT was 2.164 (95% CI, 1.111 to 5.525).

Conclusions: The Val158Met polymorphism of COMT is associated with the PSA-progression-free rate of EMP-treated patients in prostate cancer.

Keywords: Single nucleotide polymorphism; Catechol-*O*-methyltransferase; Estramustine phosphate; 2-methoxyestradiol; Prostate cancer

Abbreviations: EMP, Estramustine phosphate; COMT, Catechol-*O*-methyltransferase; EaM, Estramustine; EoM, Estromustine; 2-ME2, 2-methoxyestradiol; Val, Valine; Met, Methionine; PSA, Prostate-specific antigen; JG-SNP, Japanese single nucleotide polymorphisms for geriatric research; RFLP, Restriction fragment length polymorphism; HWE, Hardy-Weinberg equilibrium; OR, Odds ratio; CI, Confidence interval; HR, Hazard ratio; AR, androgen receptor.

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doi:10.1016/j.eururo.2005.07.007

ELSEVIER

1. Introduction

Estramustine phosphate (EMP) is a prodrug consisting of 17β-estradiol bound to nor-nitrogen mustard. The anticancer activity of EMP has been reported to be mediated by its two major metabolites, estramustine (EaM) and estromustine (EoM) [1,2], and EaM possesses more potent anticancer activity than EoM [3]. Despite its usefulness as a second-line hormonal drug for prostate cancer, EMP often causes severe side effects that reduce the patient compliance with the drug. Therefore, we have been involved in attempts to administer low-dose EMP therapy for prostate cancer, to reduce the incidence of the drug's side effects while maintaining its therapeutic efficacy.

Recently, the antiproliferative and anti-angiogenic properties of 2-methoxyestradiol (2-ME2) has drawn attention [4], and randomized phase 2 clinical trials of this drug for hormone-refractory prostate cancer were conducted in the USA. The drug was found to be well tolerated and contributed to the decline and stabilization of PSA levels in patients of prostate cancer [5]. 2-ME2, a metabolite of 17β-estradiol, which is yielded by sequential hydroxylation, and *O*-methylation of 17β-estradiol at the 2-position, is synthesized naturally in the human body.

Catechol-*O*-methyltransferase (COMT) catalyzes the *O*-methylation of 2-hydroxyestradiol to yield 2-ME2 [4]. COMT is an important enzyme that is involved in the inactivation of endogenous catecholamines and catechol drugs. COMT activity is governed by a functional single-nucleotide polymorphism (SNP) due to a G-to-A transition at codon 158 of *COMT*, resulting in a valine (Val)-to-methionine (Met) substitution. Homozygosity for 158Met leads to a 3- to 4-fold reduction in the activity of this enzyme due to increase of its thermolability [6]. Then, we considered that the enzymatic activity of COMT might be associated with both the elaboration of 2-ME2 and the clinical efficacy of EMP therapy in cases of prostate cancer. In this study, we investigated the relationship between the Val158Met polymorphism of *COMT* and PSA-progression-free survival of EMP-treated patients in prostate cancer.

2. Material and methods

2.1. Study design and treatment plan

Seventy-two Japanese patients with previously untreated prostate cancer, aged from 55 to 89 years (mean ± SD, 72.4 ± 8.0) and an Eastern Cooperative Oncology Group performance status [7] of 0 to 2, who were considered to be eligible for low-dose EMP therapy (280 or 140 mg/day) were enrolled for the study (Table 1), which was conducted from January 1999 to August 2003. These patients did not have significant active concurrent medical illness or malignancy precluding EMP treatment. Patients who had a history of cardiovascular event were excluded from this project. The tumor status of the patients was evaluated from digital rectal examination, transrectal ultrasonography, and pelvic computed tomography and was conducted according to the 2002 TNM staging system for cancer [8]. Low-dose EMP therapy was performed at the University of Tokyo Hospital and our affiliated hospitals. Thirty-two patients were treated with oral EMP alone (280 mg/day), and the remaining 40 patients were treated with oral EMP (280 mg/day in 9 patients and 140 mg/day in 31 patients) plus injection of luteinizing hormone releasing hormone agonist or surgical castration. We assessed their physical condition and serum prostate-specific antigen (PSA) levels monthly. Biochemical PSA failure was defined as a rise in the PSA level to greater than 4.0 ng/mL on three consecutive measurements after achieving a nadir, and the date of the first determination of a PSA level of 4.0 ng/mL was considered as the time-point of biochemical PSA failure. Follow-up period was 1.46 ± 1.24 (mean ± SD) years. The study was conducted with the approval of the Ethics Committee of the University of Tokyo, and after obtaining written informed consent from each of the patients prior to their entering the study.

We also examined age-matched and residence-matched 110 Japanese men as a non-cancer group to consider the distribution of *COMT* genotypes and prostate cancer susceptibility, aged from 53 to 89 years (mean ± SD, 74.1 ± 6.4), who died of cancer unrelated cause at Tokyo Metropolitan Geriatric Medical Center. Those patients had been registered in the database of Japanese single nucleotide polymorphisms for geriatric research (JG-SNP) [9]. All of them were consecutively autopsied and we confirmed pathologically that they did not carry any malignancies. Written informed consent was obtained either from the patients themselves prior to death or from family members under the Act of Postmortem Examination. The study was also reviewed and approved by the ethics committee of Tokyo Metropolitan Geriatric Medical Center.

2.2. Genotyping assays

Genotyping of the Val158Met polymorphism of *COMT* was conducted by the polymerase chain reaction (PCR)-based restriction fragment length polymorphism (RFLP) method. Genomic DNA was extracted from the peripheral blood. The concentration of the DNA solution was adjusted to 100 ng/μL before using it for PCR. Primer sequences and PCR conditions were obtained using the method described previously by Hamajima et al. [10]. Gene fragment containing the Val158Met polymorphism of *COMT* was amplified in a 50-μL volume of a solution containing 5 μL of 10 × PCR Gold Buffer, 1.5 mM $MgCl_2$, 0.2 mM dNTP (Applied Biosystems, Foster City, CA, USA), 0.5 μM each specific primer (synthesized by Fasmac, Atsugi, Kanagawa, Japan), 1.25 U of AmpliTaq Gold DNA polymerase (Applied Biosystems, Branchburg, NJ, USA), and 100 ng of genomic DNA. Twenty μL of the amplicons were digested by 5 units of *Nla*β (New England Biolabs, Beverly, MA, USA) for 3 hours at 37 °C, and the gene fragments were examined on a 3% agarose gel by ethidium bromide staining. The genotypes were distinguished as follows, 114-, 36-, and 35-bp for Val/Val *COMT*, 114-, 96-, 36-, 35- and 18-bp for Val/Met *COMT*, and 96-, 36-, 35- and 18-bp for Met/Met *COMT* (Fig. 1).

In addition, we verified the results of RFLP method by a TaqMan assay. We performed a TaqMan assay by using ABI PRISM 7000 or 7300 sequence detection system (Applied Biosystems, Foster City, CA, USA), according to the manufacturer's instructions. Sequences of specific primers and TaqMan probes,

Table 1
Characteristics of patients

	Overall	Val158Met genotype of *COMT*		*p*-value
		Val/Val	Val/Met or Met/Met	
No. of patients	72	26	46	–
Age, years				
Mean ± SD[a]	72.4 ± 8.0	74.7 ± 7.3	71.1 ± 8.1	0.063
Range	55 to 89	60 to 89	55 to 85	
Tumor status				
T1	1 (1%)	0 (0%)	1 (2%)	0.283
T2	9 (13%)	5 (19%)	4 (9%)	
T3	35 (49%)	14 (54%)	21 (46%)	
T4	27 (38%)	7 (27%)	20 (43%)	
Gleason sum				
2–7	41 (57%)	17 (65%)	24 (52%)	0.274
8–10	31 (43%)	9 (35%)	22 (48%)	
Treatment				
EMP 140 mg/day + LHRH[b] analog	31 (43%)	10 (38%)	21 (46%)	0.607
EMP 280 mg/day	32 (44%)	14 (54%)	18 (39%)	
EMP 280 mg/day + LHRH analog	4 (6%)	1 (4%)	3 (7%)	
EMP 280 mg/day + Surgical castration	5 (7%)	1 (4%)	4 (9%)	
PSA baseline level (ng/mL)[c]				
Mean ± SD[a]	53.6 ± 2.8	71.8 ± 2.2	45.5 ± 3.0	0.340
Range	0.3 to 5972.1	7.1 to 2773.8	0.3 to 5972.1	

[a] Standard deviation.
[b] Luteinizing hormone releasing hormone.
[c] Data are geometric means; SDs calculated from log2-transformed distribution.

and conditions of quantitative real-time PCR were obtained from the website of National Cancer Institute (Cancer Genome Anatomy Project SNP500Cancer Database; http://snp500cancer.nci.nih.gov/snp.cfm?both_snp_id=COMT-01).

2.3. Statistical analysis

The χ^2-test was conducted to examine the Hardy-Weinberg equilibrium (HWE) and to compare the distribution of tumor status, Gleason sum, and treatment among the genotypes. Using unpaired *t*-test compared age and the logarithmic transformed PSA baseline levels among the genotypes. Because the distribution of PSA baseline levels was skewed, we used the logarithmic transformation to estimate geometric mean of PSA baseline levels. Odds ratios (ORs) with 95% confidence intervals (CIs) for prostate cancer susceptibility were calculated by a standard Woolf-Haldane analysis. The primary end point of this study was biochemical PSA-progression-free survival. Survival was measured from the date of starting low-dose EMP therapy to the date of biochemical PSA failure. PSA-progression-free survival curves were drawn by the Kaplan-Meier method and statistical significance was analyzed by the log-rank test [11]. The effect of the COMT polymorphism on PSA levels was evaluated not only by genotype but also allele wise. We estimated the 95% CI and hazard ratio (HR) by the Cox proportional hazard model [12]. The HRs in the multivariate analysis were obtained with adjustment for Val158Met genotype of *COMT*, age, tumor status, Gleason sum, treatment, and PSA baseline level. These statistical analyses were conducted using the JMP software, version 4.0.5 (SAS, Cary, NC, USA). The significance level used was $p < 0.05$ in all statistics.

3. Results

Overall toxicity rate was as high as 66.7%. Nineteen of 72 patients (26.4%) withdrew EMP therapy because of side effects (gastrointestinal toxicity, 11 patients; hepatic toxicity, 4 patients; peripheral edema, 3 patients; arrhythmia, 1 patient). Thirteen patients (18.1%) resulted in biochemical PSA failure. New metastatic regions were not observed in all patients, however, 2 patients developed local enlargement of prostate tumor without biochemical PSA failure. No patients died of prostate cancer or side effect of EMP during the observation period.

We divided the patients by the Val158Met genotype of *COMT*, and verified their background data for comparability. No significant differences were seen among the case groups in terms of the age, tumor status, Gleason sum, treatment, or PSA baseline level (Table 1). Table 2 shows the distribution of each genotype of *COMT* both in the case group and the non-cancer group. No significant difference was observed between age distribution of the case group and that of the non-cancer group ($p = 0.105$). The allele frequencies of Val and Met were 0.653 and 0.347 in the case group, and 0.655 and 0.345 in the non-cancer

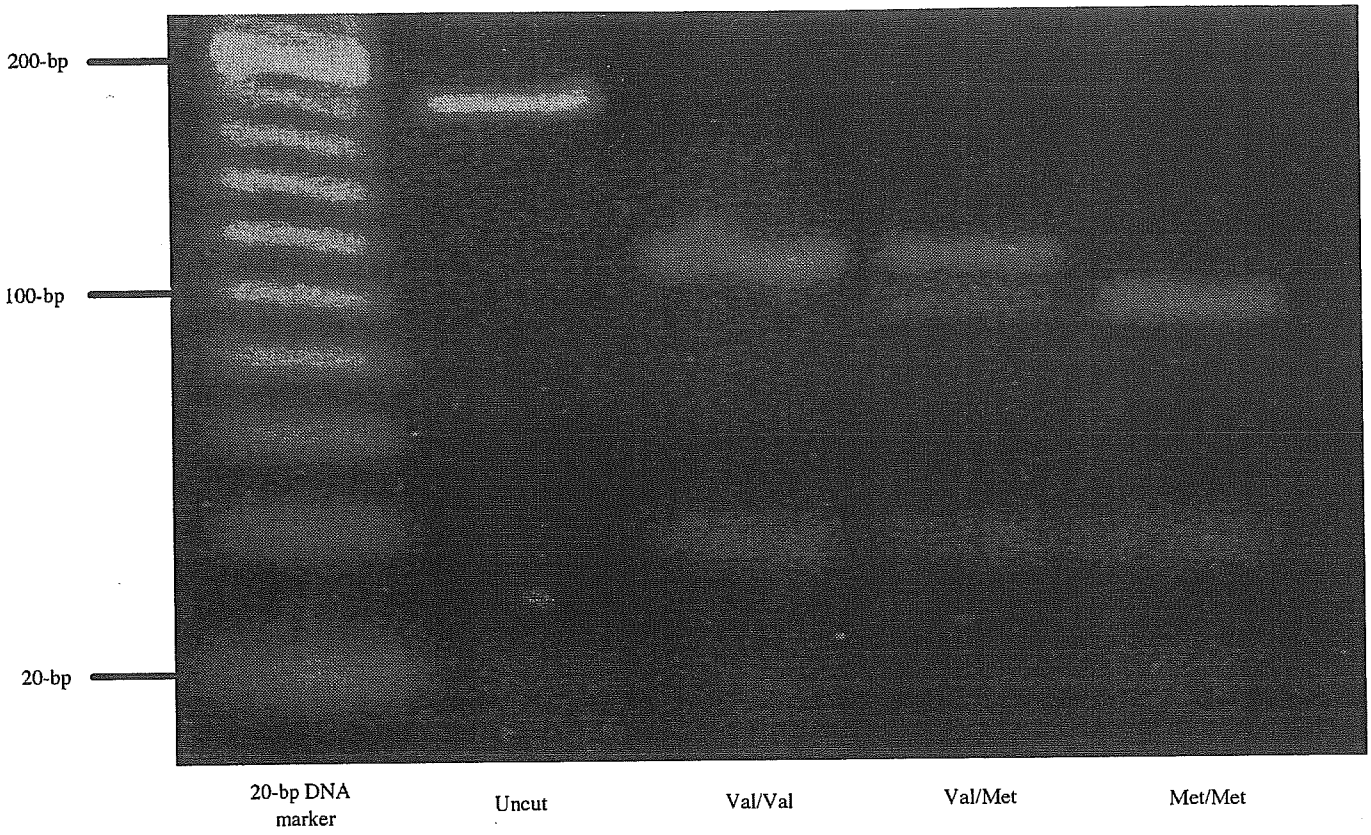


Fig. 1. The electrophoregram shows band pattern of each genotype of the Val158Met polymorphism of *COMT*. Each genotype was distinguished as follows: 185-bp for undigested, 114-, 36-, and 35-bp for the Val/Val genotype, 114-, 96-, 36-, 35-, and 18-bp for the Val/Met genotype, and 96-, 36-, 35-, and 18-bp for the Met/Met genotype.

group, respectively. The allele frequencies of Val158-Met polymorphism of *COMT* in the case and the non-cancer groups were not statistically significant ($p = 0.596$). Although the distribution of the Val158-Met genotype of *COMT* in the non-cancer group did not deviate from the HWE ($\chi^2 = 0.017$, $p = 0.897$), that in the case group deviated from the HWE ($\chi^2 = 5.922$, $p = 0.015$). The Val158Met genotype of *COMT* was not associated with prostate cancer susceptibility (Table 2).

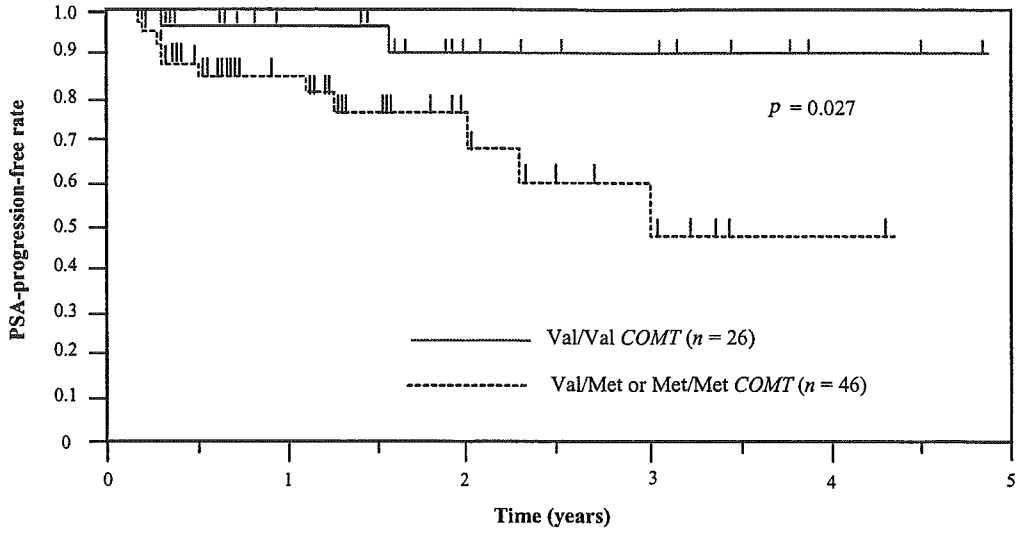
We compared the PSA-progression free survival between patients with Val/Val genotype and that with Val/Met and Met/Met genotypes, because the enzymatic activities of Val/Met and Met/Met COMT are significantly lower than that of Val/Val COMT [13].

Table 2
Prostate cancer susceptibility and HWE tests

Val158Met genotype of *COMT*		No. of patients (%)		OR[a] (95%CI[b])	*p* -value
		Case	Non-cancer		
Val/Val		26 (36.1)	47 (42.7)	1.0[d]	
Val/Met		42 (58.3)	50 (45.5)	0.659 (0.351 to 1.237)	0.194
Met/Met		4 (5.6)	13 (11.8)	1.798 (0.531 to 6.082)	0.346
Val/Met or Met/Met		46 (63.9)	63 (57.3)	0.758 (0.411 to 1.397)	0.701
Allele frequency	Val	0.653	0.655		
	Met	0.347	0.345		
HWE[c]	χ^2	5.922	0.003		
	p -value	0.015	0.957		

[a] Odds ratio.
[b] Confidence interval. 95%.
[c] Hardy-Weinberg equilibrium.
[d] Reference.

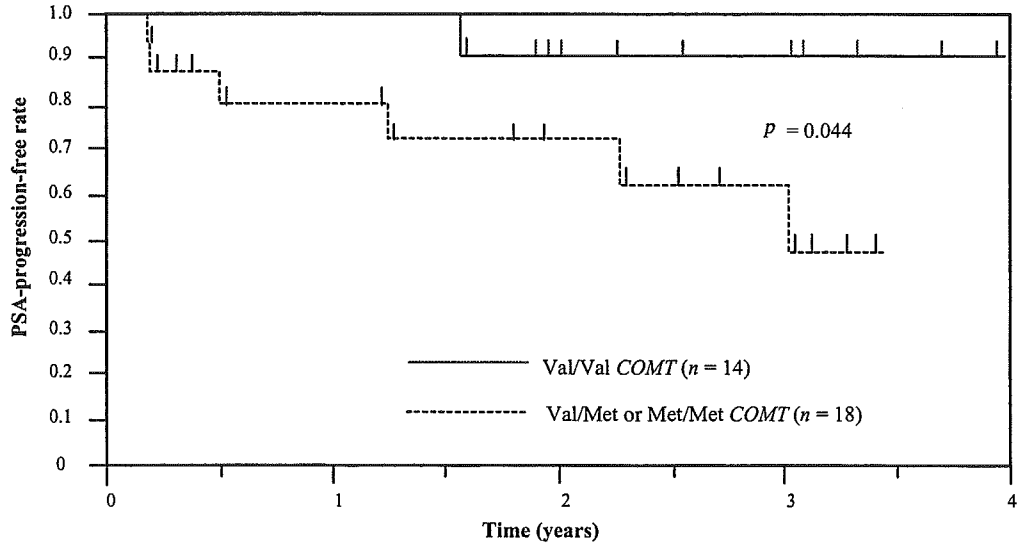
Overall



Patients under observation:

	0-year	1-year	2-year	3-year	4-year
Val/Val genotype	26	18	11	7	4
Val/Met or Met/Met genotype	46	22	9	5	1

Restricted analysis



Patients under observation:

	0-year	1-year	2-year	3-year	4-year
Val/Val genotype	14	13	9	5	1
Val/Met or Met/Met genotype	18	11	8	4	0

Fig. 2. Estimated PSA-progression-free survival curves by the Kaplan-Meier method.Vertical marks represent censored patients. Patients with the Val/Val genotype of *COMT* had a significantly higher PSA-progression-free survival than patients with the Val/Met or Met/Met genotype.

Patients with the Val/Val genotype of *COMT* had a significantly higher PSA-progression-free survival than patients with the Val/Met or Met/Met genotype (log-rank test, $p = 0.027$) (Fig. 2). The 3-year PSA-progression-free survival was 89.4% in the group with the Val/Val genotype and 47.2% in the group with the Val/Met or Met/Met genotype. When we restricted our analysis to only patients receiving oral EMP at the dose of 280 mg/day, the PSA-progression-free survival was also significantly higher in the group with the Val/Val genotype than in that with the Val/Met or Met/Met genotype (log-rank test, $p = 0.044$). The 3-year PSA-progression-free survivals were 91.7% in the group with the Val/Val genotype and 46.7% in the group with the Val/Met or Met/Met genotype. In addition, we evaluated the effect of the COMT polymorphism on PSA levels by allele wise. With whole 72 cases (144 alleles), the Val allele had a slightly higher PSA-progression-free survival than the Met allele (log-rank test, $p = 0.083$). When we restricted our analysis to only subjects receiving oral EMP at the dose of 280 mg/day (64 alleles), the PSA-progression-free survival was significantly higher in the Val allele than in the Met allele (log-rank test, $p = 0.028$).

The findings of the univariate and the multivariate analyses showed that the Val158Met genotype of *COMT* was a significant factor for the survival of biochemical PSA failure. The adjusted hazard ratio of the Val158Met genotype of *COMT* was 4.784 (95% CI, 1.226 to 31.567) (Table 3).

4. Discussion

We have previously demonstrated the efficacy of low-dose EMP monotherapy in patients with prostate cancer [14]. Overall PSA response rate of our study was as high as 93.4% that was almost equal to the outcome of EMP therapy at the dose of 560 mg/day for previously untreated advanced prostate cancer [14]. In contrast, overall toxicity rate was 39.5% in EMP therapy at the dose of 280 mg/day and 55.0% in that at the dose of 560 mg/day, respectively [14,15]. Recent our data also suggested the incidence of side effect of EMP increased in a dose-dependent manner [16].

The actual mechanism underlying the efficacy of low-dose EMP therapy for prostate cancer remains to be clearly established. Our data suggested the Val158-Met polymorphism of *COMT* is associated with the PSA-progression-free survival of EMP-treated patients in prostate cancer. The difference of enzymatic activity of COMT contributes to both 17β-estradiol metabolism and 2-ME2 elaboration. Wang et al. suggested that EaM and EoM exhibit their antagonistic effects on both mutated androgen receptor (m-AR) and wild-type AR, whereas hydroxyflutamide and 17β-estradiol act as agonists to m-AR [17]. Considering that 17β-estradiol has agonistic property for m-AR during EMP therapy, patients with Val/Val genotype of *COMT* may be favorable to reduce agonistic effect of 17β-estradiol. On the other hand, considering that 2-ME2 has anti-proliferative and anti-angiogenic properties [4],

Table 3
Univariate and multivariate analyses of biochemical PSA failure prediction

Factor	Univariate			Multivariate		
	HR[a]	95% CI[b]	*p*-value	HR	95% CI	*p*-value
Val158Met genotype of *COMT*						
Val/Val	1.0[c]			1.0[c]		
Val/Met or Met/Met	4.709	1.251 to 30.592	0.020	4.784	1.226 to 31.567	0.022
Age	0.947	0.879 to 1.016	0.131	0.965	0.892 to 1.039	0.342
Tumor status						
T1-T3	1.0[c]			1.0[c]		
T4	2.478	0.823 to 7.698	0.105	1.928	0.597 to 6.373	0.268
Gleason sum						
2–7	1.0[c]			1.0[c]		
8–10	1.327	0.425 to 4.019	0.615	0.966	0.286 to 3.161	0.954
Treatment						
EMP alone	1.0[c]			1.0[c]		
Combined therapy	1.201	0.369 to 3.823	0.754	0.798	0.225 to 2.806	0.720
Log2 (PSA baseline level)	1.223	0.988 to 1.505	0.065	1.212	0.998 to 1.462	0.052

[a] Hazard ratio.
[b] Confidence interval, 95%.
[c] Reference.

patients with Val/Val genotype of *COMT* may also be favorable to elaborate 2-ME2. 2-ME2 was found to be capable of inhibiting the proliferation of DU145 (a human prostate cancer cell line) cells more effectively than EaM and EoM. The 50%-inhibitory concentrations of 2-ME2, EaM and EoM against the cellular proliferation of DU145 cells have been reported to be 1.8, 20.5 and 65.6 μmol/L, respectively [3,18]. There remains a need to compare the serum 17β-estradiol and 2-ME2 levels by genotypes of the Val158Met polymorphism of *COMT*.

In our study, the distribution of the Val158Met genotype of *COMT* in our patients deviated from the HWE. There is one interesting paper reported by Suzuki et al. [19]. They evaluated the association between the Val158Met polymorphism of *COMT* and the risk of developing familial prostate carcinoma in Japanese population. Their data showed the distributions of Val/Val, Val/Met, and Met/Met were 38 (37.6%), 55 (54.5%), and 8 (7.9%) in patients with familial prostate cancer and 52 (45.6%), 51 (44.7%), and 11 (9.6%) in the non-cancer group, respectively. To examine their data for the HWE by the χ^2 test, the p values were 0.051 in the case group and 0.768 in the non-cancer group. The distribution of the Val158Met genotype of COMT in the case group that Suzuki et al. reported was barely in the HWE. Our data suggest a disparity of the HWE between the case group and the non-cancer group. To confirm our data, the HWE test should be performed with more number of cases in the future.

A weak point of this study was short observation period. We thought that high withdrawal rate of EMP therapy made the observation period shorten. If we can select the patients who are tolerable to EMP's toxicities, the contribution of the Val158Met polymorphism of *COMT* to survival will be analyzed more accurately.

We first demonstrated that the Val158Met polymorphism of *COMT* is associated with the PSA-progression-free survival of EMP-treated patients in prostate cancer. Further studies are needed to clarify the clinical value of the Val158Met polymorphism of *COMT* in terms of prognosis and the drug properties of EMP.

5. Conflict of interest

This study was supported by a Grant-in-Aid from the Japanese Ministry of Education, Science, Sports and Culture (Project No. 15390485) and a grant from Yamaguchi Endocrine Research Association. The sponsors of the study had no role in study design, data collection, data analysis, data interpretation, writing of the report, or in decision on submit the manuscript for publication.

Acknowledgements

The authors thank all the investigators who contributed to this project, especially AM Treston for their editorial assistance, A Hirose and E Tanaka for their excellent technical assistance, and H Nishimatsu and C Kato for their data collection.

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Human Molecular Genetics, 2006, Vol. 15, No. 7 *1151–1158*
doi:10.1093/hmg/ddl030
Advance Access published on February 24, 2006

Multiple candidate gene analysis identifies α-*synuclein* as a susceptibility gene for sporadic Parkinson's disease

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Received December 22, 2005; Revised and Accepted February 15, 2006

Parkinson's disease (PD), one of the most common human neurodegenerative diseases, is characterized by the loss of dopaminergic neurons in the substantia nigra of the midbrain. PD is a complex disorder with multiple genetic and environmental factors influencing disease risk. To identify susceptible genes for sporadic PD, we performed case–control association studies of 268 single nucleotide polymorphisms (SNPs) in 121 candidate genes. In two independent case–control populations, we found that a SNP in α-*synuclein* (*SNCA*), rs7684318, showed the strongest association with PD ($P = 5.0 \times 10^{-10}$). Linkage disequilibrium (LD) analysis using 29 SNPs in a region around rs7684318 revealed that the entire *SNCA* gene lies within a single LD block ($D' > 0.9$) spanning ~120 kb. A tight LD group ($r^2 > 0.85$) of six SNPs, including rs7684318, associated most strongly with PD ($P = 2.0 \times 10^{-9}$–1.7×10^{-11}). Haplotype association analysis did not show lower *P*-values than any single SNP within this group. SNCA is a major component of Lewy bodies, the pathological hallmark of PD. Aggregation of SNCA is thought to play a crucial role in PD. *SNCA* expression levels tended to be positively correlated with the number of the associated allele in autopsied frontal cortices. These findings establish *SNCA* as a definite susceptibility gene for sporadic PD.

INTRODUCTION

Sporadic Parkinson's disease (PD) (OMIM no. 168600) is the second most common neurodegenerative disease following Alzheimer's disease. PD is late onset and progressive, affecting 1–2% of persons older than 65 years. Clinical features of PD include resting tremor, bradykinesia, rigidity and postural instability. The disease is pathologically characterized by the loss of dopaminergic neurons in the substantia nigra and the presence of intracellular inclusions known as Lewy bodies. Various medical managements are available for PD, including drugs (l-dopa, dopamine agonists, anti-cholinergic drugs, etc.) and surgery (thalamotomy, pallidotomy, deep brain stimulation, etc.) (1). These treatments improve PD symptoms, but do little to deter disease progression. Identifying risk factors for PD can be helpful in delaying disease onset and slowing its progression.

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PD is a complex common disease, caused by multiple genetic and environmental factors (2). The contribution of genetic factors to sporadic PD is indicated by several findings. First, ~10% of patients with PD have a positive family history (3). Secondly, a recent large-scale survey in Iceland showed that the risk ratio for PD was increased in related individuals (6.7 for siblings, 3.2 for offspring and 2.7 for nephews and nieces of patients with PD) (4). Thirdly, a twin study using [^{18}F]dopa PET showed that the concordance rate for PD, including subclinical cases, is approximately three times higher in monozygotic twins (55%) than in dizygotic twins (18%) (5).

Causal genes for Mendelian-inherited PD have been reported, including *α-synuclein* [4q21, autosomal dominant (AD)] (6), *parkin* [6q25.2–27, autosomal recessive (AR)] (7), *UCH-L1* (4p14, AD) (8), *PINK1* (1p36, AR) (9), *DJ-1* (1p36, AR) (10), *LRRK2/dardarin* (12q12, AD) (11,12) and *NR4A2/Nurr1* (2q22–23, AD) (13).

Many case–control association studies using single nucleotide polymorphisms (SNPs) in candidate genes have been reported, but few consistent findings have been obtained (2). This is due, in part, to limited numbers of available samples, target genes and/or genetic markers. Since 2001, genome-wide, non-parametric linkage analysis of PD families has revealed significant linkage in multiple chromosomal regions (14–17), leading to the identification of *tau* (18) and *FGF20* (19) as susceptibility genes.

To date, polymorphisms that influence PD as strongly as *APOE-ε4* influences Alzheimer's disease have not been identified. Through extensive candidate gene association studies, we have established *α-synuclein* (*SNCA*) as a definite susceptibility gene for sporadic PD.

RESULTS

Screening of SNPs in candidate genes for PD

We selected candidate genes from the literature describing genetic, pathological and biochemical findings in PD, as well as genes that participate in the proposed mechanisms for PD. Finally, we picked up 121 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 (268 SNPs total) were selected from the dbSNP, JSNP and Celera Discovery System databases.

In the initial screen, we genotyped 190 patients and 190 controls (Supplementary Material, Table S1). To avoid false negatives, we set the α-value at 0.05 in the first screen. From 268 SNPs, 22 SNPs in 16 genes showed association with PD ($P < 0.05$) in genotype frequency, allele frequency, dominant model or recessive model. We genotyped the 22 qualifying SNPs in a replication panel of 692 patients and 748 controls and tested again for association. This independent test revealed that SNP0070 (rs7684318 C/T) was prominently associated with PD ($P = 5.0 \times 10^{-10}$ for allele frequency) (Table 1). We corrected the α-value to 0.00019 after Bonferroni's correction (tests for 268 SNPs). The remaining 21 SNPs did not show P-values lower than 0.00019 (data not shown). SNP0070 is located in intron 4 of the *α-synuclein* (*SNCA*) gene on chromosome 4q21. SNCA is a primary component of intracellular inclusions called Lewy bodies, which are considered to be the pathological hallmark of PD (20). Aggregation of SNCA is thought to play a crucial role in the pathogenesis of PD (21). The allele C frequency of SNP0070 was higher in PD (0.67) than in controls (0.57) (Table 1). The association of SNP0070 was significant in genotype frequency, allele frequency, dominant model and recessive model. Of the two disease models, allele C of SNP0070 was more significantly associated in the recessive model than in the dominant model (Table 1).

Linkage disequilibrium (LD) mapping and search for susceptibility SNPs

We performed LD mapping in a 430 kb region around SNP0070. This region contains two genes: *SNCA* and *MMRN1*. Using SNP0070 and 28 additional SNPs in this region, we genotyped 134 control subjects and constructed an LD map based on pairwise D' and r^2 (Fig. 1) (Supplementary Material, Table S2). Three LD blocks were observed on the basis of D' ($D' > 0.9$). The entire *SNCA* gene was included in a block containing SNP0070 (block 2). The *MMRN1* gene was in another LD block, indicating that *MMRN1* does not correlate with the SNP0070 association (Fig. 2).

To search for the most strongly associated SNP(s) in the region, we next performed association studies with these 29 SNPs (Fig. 2; Table 2). We found significant associations for SNPs in block 2, but not in blocks 1 and 3. Block 2, thought to be a susceptibility block for PD, was further analyzed on the basis of r^2-values. Of the 19 SNPs in block 2, 16 belonged to three groups with high pairwise r^2 (>0.85) and the remaining three did not belong to any group (Fig. 1; Table 2) (Supplementary Material, Table S2). Six SNPs in group 1, including originally screened SNP0070 and five additional SNPs (0203, 0204, 0205, 0207 and 0209), showed prominent association with PD ($P = 2.0 \times 10^{-9}$–1.7×10^{-11}, allele 1 versus allele 2) (Fig. 2; Table 2). Population attributable risk (PAR) (22) of SNP0070 was 42.5% in the dominant model and 18.5% in the recessive model.

We next performed haplotype analysis using six representative SNPs in block 2 (Table 3). Six common haplotypes (>1% of PD and controls) covered >90% of the population haplotypes in both PD and controls. The major haplotypes 1 and 2 showed significant associations; however, their P-values were not lower than that of any single SNP in group 1. Therefore, the presence of hidden SNP(s) with a lower P-value than group 1 seemed unlikely, as was the possibility that the haplotype(s) is implicated in PD susceptibility. These findings establish the six SNPs in group 1 as the strongest susceptibility SNPs. All showed stronger associations in the recessive model than in the dominant model, similar to the originally screened SNP0070 (Table 4).

Taken together, our genetic analyses indicate that *SNCA* is a definite susceptibility gene for sporadic PD and that multiple SNPs in group 1 are susceptibility SNPs, likely in a recessive model.

Table 1. Association of SNP0070 in *SNCA* between cases and controls

	Genotype				Allele			*P*-value (χ^2-test)			
	CC	CT	TT	Total	C	T	Total	Genotype	Allele	Dominant[a] model	Recessive[b] model
First screen											
Case	87 (0.46)	87 (0.46)	14 (0.07)	188	261 (0.69)	115 (0.31)	376	3.4×10^{-4}	1.8×10^{-4}	1.8×10^{-4}	1.1×10^{-2}
Control	62 (0.33)	85 (0.46)	39 (0.21)	186	209 (0.56)	163 (0.44)	372				
Replication											
Case	298 (0.44)	307 (0.45)	75 (0.11)	680	903 (0.66)	457 (0.34)	1360	1.3×10^{-6}	4.2×10^{-7}	1.5×10^{-3}	9.0×10^{-7}
Control	233 (0.31)	387 (0.52)	126 (0.17)	746	853 (0.57)	639 (0.43)	1492				
Total											
Case	385 (0.44)	394 (0.45)	89 (0.10)	868	1164 (0.67)	572 (0.33)	1736	2.7×10^{-9}	5.0×10^{-10}	5.7×10^{-6}	2.8×10^{-8}
Control	295 (0.32)	472 (0.51)	165 (0.18)	932	1062 (0.57)	802 (0.43)	1864				

Frequencies of genotypes and alleles are in parentheses.
[a]Genotype CC+CT versus TT.
[b]Genotype CC versus CT+TT.

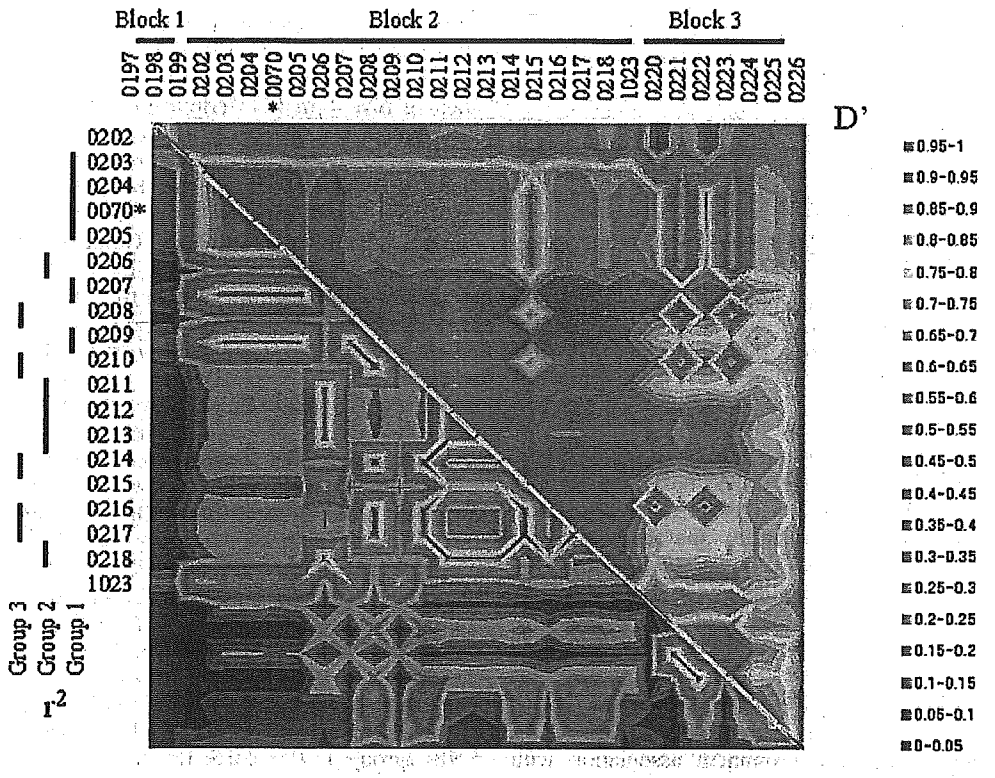

Figure 1. LD structure of the susceptibility region for sporadic PD. Pairwise LD between SNPs, as measured by D' in 134 controls, is graphically indicated. The region spanning 430 kb around the originally screened SNP0070(*) was divided into three LD blocks ($D' > 0.9$) (upper right). On the basis of r^2, SNPs in block 2, including SNP0070, were further divided into three groups ($r^2 > 0.85$) and three solitary SNPs (lower left). The scale is nominal.

SNCA gene expression in relation to susceptibility genotypes

To examine whether the strongest associated SNPs (group 1) affect *SNCA* gene expression, we further quantified *SNCA* mRNA in autopsied frontal cortices and compared the values among the genotypes. SNP0070, in which allele C is associated with PD, was used as a representative of group 1.

The relative values of *SNCA* mRNA for all cases ($n = 21$) and all controls ($n = 18$) were 1.07 ± 0.10 and 0.95 ± 0.13, respectively, showing almost the same level ($P = 0.46$, Student's t-test). When compared among the genotypes in cases, the mean tended to decrease in the order of CC, CT and TT (Fig. 3), although the differences did not reach the significant levels ($P = 0.71$ for CC versus CT, $P = 0.16$ for CT versus TT and $P = 0.32$ for CC versus TT). Similar tendency

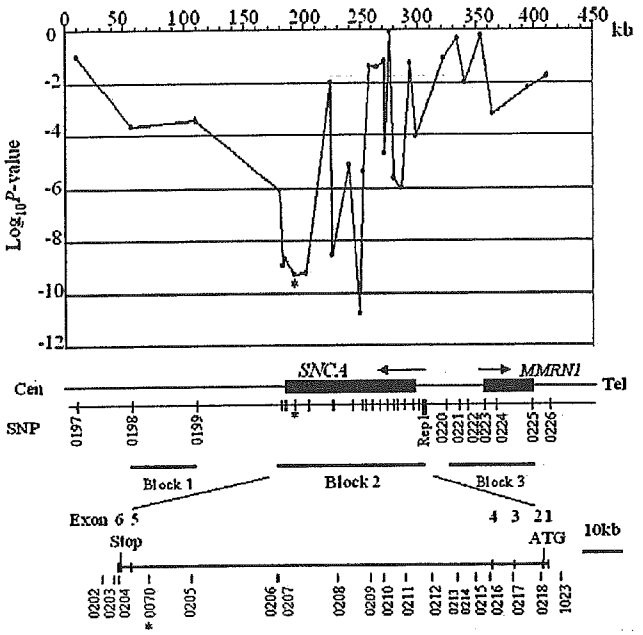

Figure 2. Genomic structure and SNPs of the susceptibility region for sporadic PD and case–control association studies (882 cases and 938 controls). Log *P*-values (allele 1 versus allele 2) are plotted against the physical location of the SNPs. The region includes two genes: *SNCA* and *MMRN1*; transcription orientation is indicated by horizontal arrows. Physical locations of SNPs are shown as axial bars with our experimental ID number. The originally screened SNP0070 is indicated by an asterisk. The location of Rep1, a well-known repeat polymorphism in the *SNCA* promoter region, is indicated by a thick bar. SNPs in block 2 are nominated in an expanded map with the exon–intron structure of *SNCA*. SNPs in group 1 are shown in red. Note that *P*-values are prominently low at the group 1 SNPs located in the 3′ region of *SNCA*. *P*-values in the region around Rep1 are far from significant when compared with those in group 1.

was observed in controls. The mean tended to decrease in the order of CC, CT and TT (Fig. 3) ($P = 0.33$ for CC versus CT, $P = 0.59$ for CT versus TT and $P = 0.54$ for CC versus TT).

These results indicate the possibility that expression of *SNCA* mRNA in the brain tends to be positively correlated with the number of PD-associated allele.

DISCUSSION

To identify susceptibility genes for PD, we performed an extensive candidate gene approach by screening 268 SNPs in 121 genes and identified a prominent association with SNP0070 (rs7684318) in the *SNCA* gene (Table 1). LD mapping localized the entire *SNCA* gene within a single LD block (Figs 1 and 2). Within this block, six SNPs including SNP0070 were in a tight LD group and most strongly associated with PD (Fig. 2; Table 2). The major allele of each SNP in group 1 was positively associated with PD, more strongly in the recessive model than in the dominant model (Table 4). Our genetic analyses establish *SNCA* as a definite susceptibility gene for PD and identify multiple SNPs in group 1 as susceptibility SNPs. Recently, Mueller *et al.* (23) reported that multiple regions of *SNCA* are associated with PD in the German population. Associated SNPs identified by Mueller *et al.* included rs356165 ($P = 1.5 \times 10^{-4}$), which corresponds to SNP0204 in our study, indicating that this SNP has a similar association in Caucasians. Pals *et al.* (24) previously reported no association of the haplotype containing rs356165 with PD in Belgian samples. This contradictory finding may be, at least in part, due to a small sample size (175 cases and 186 controls), as mentioned by the authors.

SNCA/α-synuclein was originally identified in the electric organ of the Pacific electric ray (25). SNCA is a presynaptic protein that is highly and broadly expressed in the brain, but its normal function remains unknown (21). It is a major component of Lewy bodies, the pathological hallmark of PD (20), and the aggregation of SNCA protein is thought to play a crucial role in the loss of dopaminergic neurons (21,26).

SNCA was also the first gene identified as a causative gene in familial PD. Three missense mutations in *SNCA* were reported in families with AD inheritance (6,27,28). These mutations are thought to increase the aggregation of SNCA protein. Point mutations in *SNCA* have not been identified in sporadic PD (27,29), and no SNPs have been found in the coding region, suggesting that disease-related amino acid changes in SNCA are unlikely in sporadic PD.

Genes' overdosage is a potential mechanism for the influence of *SNCA* in PD. Triplication of the *SNCA* locus has been seen in an AD PD family (30), and doubling of *SNCA* gene dosage by triplication has been shown to result in the doubling of mRNA and protein expression in blood and brain (31). Duplication of *SNCA* has also been identified as a cause of familial PD (32,33). Clinical features of patients with *SNCA* duplication resemble those of sporadic cases and are much milder than those with triplication. Taken together, these observations indicate a correlation between increased SNCA protein levels and disease risk. Identification of one or more polymorphisms related to *SNCA* expression level might reveal strong susceptibility indicators for sporadic PD. Many studies have focussed on a mixed repeat microsatellite polymorphism called Rep1 (34), because of its location in the *SNCA* promoter region. However, their significance is uncertain, possibly because of the small number of samples (35–37). Our study demonstrates that the *P*-values of SNPs around Rep1 (0218, 1023 and 0220) are less significant than that of the SNPs in group 1 (Fig. 2). In addition, we genotyped our samples for Rep1. Pairwise *D′*-values showed that Rep1 was not in block 2, but on the boundary (Supplementary Material, Table S2). *P*-value of Rep1 was 7.5×10^{-7} (Supplementary Material, Table S3), which might be explained by its intermediate correlation with the strongest susceptibility SNPs (group 1, $P = 2.0 \times 10^{-9}$–1.7×10^{-11}). Our findings suggest that *P*-value of Rep1 depends on its LD strength with SNPs in group 1. LD strength may be modified by the unstableness of microsatellite markers (38) and may vary among races (39). Taken together, these findings may also partly explain the contradictory findings of previous Rep1 association studies.

To investigate the relationship between the SNPs in group 1 and the *SNCA* expression levels, we analyzed *SNCA* mRNA expression in autopsied frontal cortices (Fig. 3). *SNCA* expression levels tended to be positively correlated with the number of the PD-associated allele, supporting the popular hypothesis that increased SNCA leads to the disease.

Table 2. Association analysis in *SNCA* and surrounding region

SNPs			LD block	Genotype							
ID (rs ID)	Alleles 12	Location	(group)	Case 11/12/22	(Total)	Control 11/12/22	(Total)	MAF Case/control	Allele 1 versus allele 2 *P*-value	OR (95% CI)	HWE Case/control
0197 (rs3733450)	TC		1	38/286/549	(873)	33/280/619	(932)	0.21/0.19	0.10	1.15 (0.97–1.36)	1.00/0.93
0198 (rs1390280)	AG		1	366/384/118	(868)	316/454/162	(932)	0.36/0.42	2.1×10^{-4}	1.29 (1.13–1.46)	0.32/1.00
0199 (rs3733449)	CT		1	117/375/374	(866)	154/451/322	(927)	0.35/0.41	3.7×10^{-4}	1.28 (1.11–1.48)	0.16/0.91
0202 (rs356221)	TA	3′-flanking	2	73/369/431	(873)	123/449/360	(932)	0.30/0.37	7.2×10^{-7}	1.42 (1.25–1.63)	0.69/0.40
0203 (rs3857053)	TC	3′-flanking	2 (1)	380/406/87	(873)	293/476/164	(933)	0.33/0.43	1.1×10^{-9}	1.53 (1.33–1.73)	0.18/0.24
0204 (rs356165)	GA	3′-UTR	2 (1)	379/399/89	(867)	289/482/159	(930)	0.33/0.43	2.0×10^{-9}	1.52 (1.33–1.74)	0.32/0.09
0070[a] (rs7684318)	CT	Intron 4	2 (1)	385/394/89	(868)	295/472/165	(932)	0.33/0.43	5.0×10^{-10}	1.54 (1.35–1.75)	0.47/0.35
0205 (rs3775424)	CT	Intron 4	2 (1)	87/406/376	(869)	166/477/288	(931)	0.33/0.43	5.4×10^{-10}	1.52 (1.34–1.75)	0.16/0.22
0206 (rs3775426)	CT	Intron 4	2 (2)	56/350/456	(862)	53/324/555	(932)	0.27/0.23	0.0098	1.22 (1.05–1.41)	0.35/0.59
0207 (rs3796661)	CT	Intron 4	2 (1)	91/367/382	(840)	154/482/296	(932)	0.33/0.42	2.7×10^{-9}	1.52 (1.31–1.76)	0.90/0.08
0208 (rs3775435)	GA	Intron 4	2 (3)	157/434/272	(863)	115/439/375	(929)	0.43/0.36	7.3×10^{-6}	1.36 (1.18–1.56)	0.53/0.48
0209 (rs2737029)	TC	Intron 4	2 (1)	84/377/402	(863)	156/480/297	(933)	0.32/0.42	1.7×10^{-11}	1.60 (1.40–1.83)	0.81/0.12
0210 (rs3775442)	TC	Intron 4	2 (3)	158/438/274	(870)	114/440/378	(932)	0.43/0.36	4.2×10^{-6}	1.37 (1.19–1.58)	0.50/0.46
0211 (rs3756055)	GA	Intron 4	2 (2)	50/339/481	(870)	49/319/565	(933)	0.25/0.22	0.042	1.17 (1.00–1.37)	0.38/0.72
0212 (rs3775446)	TG	Intron 4	2 (2)	50/340/480	(870)	49/317/565	(931)	0.25/0.22	0.034	1.19 (1.01–1.38)	0.36/0.67
0213 (rs3756056)	CT	Intron 4	2 (2)	50/340/482	(872)	48/323/557	(928)	0.25/0.23	0.062	1.16 (0.99–1.34)	0.37/0.97
0214 (rs894278)	GT	Intron 4	2 (3)	156/438/275	(869)	117/441/375	(933)	0.43/0.36	1.9×10^{-5}	1.34 (1.18–1.52)	0.46/0.52
0215 (rs1812923)	CA	Intron 4	2	74/383/413	(870)	92/392/447	(931)	0.31/0.31	0.79	1.01 (0.89–1.16)	0.30/0.71
0216 (rs2298728)	AG	Intron 4	2 (3)	163/432/274	(869)	117/435/380	(932)	0.44/0.36	2.2×10^{-6}	1.38 (1.22–1.56)	0.80/0.72
0217 (rs3796667)	AT	Intron 3	2 (3)	159/430/271	(860)	114/428/383	(925)	0.44/0.36	9.2×10^{-7}	1.41 (1.23–1.61)	0.66/0.80
0218 (rs2035268)	TG	Intron 2	2 (2)	475/339/54	(868)	556/326/51	(933)	0.26/0.23	0.049	1.16 (0.99–1.37)	0.59/0.79
1023 (rs1023777)	CT	5′-flanking	2	66/318/464	(848)	86/433/411	(930)	0.27/0.33	9.3×10^{-5}	1.33 (1.15–1.55)	0.31/0.08
0220 (rs2736994)	GA		3	542/263/22	(827)	529/292/33	(854)	0.19/0.21	0.081	1.16 (0.98–1.38)	0.17/0.41
0221 (rs11097239)	CA		3	245/437/182	(864)	272/431/226	(929)	0.46/0.48	0.48	1.05 (0.92–1.19)	0.67/0.04
0222 (rs1899389)	AG		3	592/245/29	(866)	586/297/46	(929)	0.18/0.21	0.009	1.25 (1.05–1.46)	0.64/0.34
0223 (rs2289515)	AT		3	180/436/238	(854)	221/423/267	(911)	0.47/0.48	0.6	1.03 (0.90–1.18)	0.49/0.04
0224 (rs3775464)	GA		3	109/414/346	(869)	95/385/449	(929)	0.36/0.31	5.9×10^{-4}	1.28 (1.11–1.46)	0.43/0.40
0225 (rs1246270)	GA		3	372/394/84	(850)	474/372/81	(927)	0.33/0.29	0.0061	1.21 (1.05–1.40)	0.19/0.56
0226 (rs3822098)	CT		3	50/300/514	(864)	59/376/494	(929)	0.23/0.27	0.017	1.21 (1.04–1.40)	0.54/0.30

MAF, minor allele frequency. When the odds ratio (OR) is less than 1, an inverted score is indicated.
[a]Originally screened SNP.

Table 3. Haplotype association analysis using representative SNPs in block 2

Haplotypes	Representative SNP (group)						Haplotype frequency		*P*-value
	202	0070 (1)	0206 (2)	0214 (3)	0215	1023	Case	Control	
1	A	C	T	G	A	T	0.39	0.33	4.4×10^{-5}
2	T	T	T	T	A	C	0.24	0.3	5.0×10^{-6}
3	A	C	C	T	C	T	0.24	0.21	0.071
4	A	T	T	T	C	T	0.03	0.06	3.3×10^{-4}
5	T	T	T	T	C	T	0.02	0.03	0.083
6	T	T	T	G	A	T	0.01	0.02	0.62

Table 4. Association of the SNPs in group 1 of block 2

SNP	Allele	Genotype						Dominant model (MM + Mm versus mm)		Recessive model (MM versus Mm + mm)	
		Case			Control						
	M/m	MM	Mm	mm	MM	Mm	mm	*P*-value	Odds ratio (95% CI)	*P*-value	Odds ratio (95%CI)
0203	T/C	380	406	87	293	476	164	3.0×10^{-6}	1.95 (1.45–2.52)	1.0×10^{-7}	1.68 (1.41–2.07)
0204	G/A	379	399	89	289	482	159	2.7×10^{-5}	1.81 (1.36–2.38)	3.0×10^{-8}	1.72 (1.43–2.13)
0070[a]	C/T	385	394	89	295	472	165	5.7×10^{-6}	1.90 (1.44–2.53)	2.8×10^{-8}	1.71 (1.42–2.06)
0205	T/C	376	406	87	288	477	166	1.8×10^{-6}	1.98 (1.45–2.61)	6.0×10^{-8}	1.69 (1.40–2.05)
0207	T/C	382	367	91	296	482	154	5.3×10^{-4}	1.66 (1.25–2.16)	3.0×10^{-9}	1.78 (1.47–2.16)
0209	C/T	402	377	84	297	480	156	1.4×10^{-5}	1.89 (1.41–2.51)	1.5×10^{-10}	1.86 (1.55–2.27)

M and m are major allele and minor allele, respectively. CI, confidence interval.
[a]Originally screened SNP.

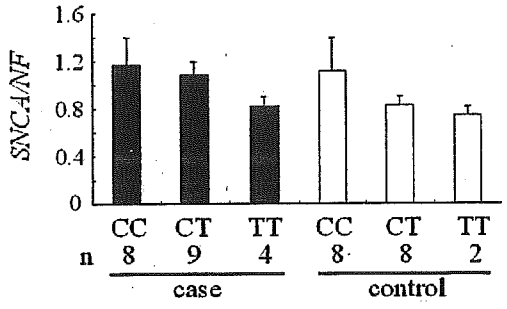

Figure 3. *In vivo* expression of *SNCA* mRNA in relation to susceptibility genotypes. SNP0070 (C/T) is used as a representative of group 1. *SNCA* expression levels in autopsied frontal cortices of cases (solid bar; 8 CC, 9 CT and 4 TT) and controls (open bar; 8 CC, 8 CT and 2 TT). Relative *SNCA* mRNA levels (normalized to *neurofilament L, NF*) are indicated. In cases, mean ± SEM of CC, CT and TT were 1.17 ± 0.23, 1.08 ± 0.11 and 0.82 ± 0.08, respectively. In controls, mean ± SEM of CC, CT and TT were 1.11 ± 0.28, 0.83 ± 0.07 and 0.75 ± 0.07, respectively.

The PD-associated alleles may positively correlate with the basal transcription level of *SNCA* and/or the induction of *SNCA* expression by certain stimulators, for example, oxidative stress.

Other possible functional effects of associated SNPs include alternative splicing, which may result in a protein isoform that aggregates more readily. The C-terminal region of SNCA is rich in acidic amino acid residues, and its truncation promotes aggregation *in vitro* (40,41). The known splice variant *SNCA112* lacks exon 5, which encodes 28 amino acids (10 of which are acidic) in frame. Thus, SNCA112 may also promote aggregation. We investigated *SNCA112* mRNA expression in frontal cortices using splice variant-specific primers, but observed little difference among the three genotypes (data not shown).

In summary, our study establishes *SNCA* as a susceptibility gene for sporadic PD. Focussed investigations of SNCA function will further enhance our understanding of how genetic factors contribute to the complex etiology of PD.

MATERIALS AND METHODS

Subjects

We recruited 882 unrelated sporadic PD patients (age, 64.9 ± 9.8; male/female ratio, 0.79) and 938 unrelated controls (age, 45.3 ± 16.3; male/female ratio, 1.10). The diagnosis of idiopathic PD was based on the presence of two or more of the cardinal features of PD (tremor, rigidity, bradykinesia and postural instability), according to the criteria for sporadic PD (42). Patients were evaluated by the certified neurologists specializing in PD. The average age of onset was 57.4 ± 10.9 years. Forty-two patients showed early onset of PD (<40 years) and 51 patients had a positive family history of PD. Patients who carried *parkin* mutations were excluded. All patients and controls were of Japanese ancestry. Informed consent was obtained from each individual, and approval for the study was obtained from the University Ethical Committees.

SNP genotyping

Genomic DNA was extracted from whole blood using FlexGene (Qiagen). SNP information was obtained from the dbSNP (http://www/ncbi.nlm.nih.gov/SNP/), JSNP (http://snp.ims.u-tokyo.ac.jp/) (43) and Celera Discovery System

(http://myscience.appliedbiosystems.com/) databases. We genotyped SNPs using the Invader assay (Third Wave Technologies), TaqMan (Applied Biosystems) or direct sequencing using an ABI3730 capillary sequencer (Applied Biosystems). Rep1 genotyping and allele designations followed those described previously (35). The Rep1 region was amplified using FAM5′-CCTGGCATATTTGATTGCAA-3′ and 5′-GACTGGCCCAAGATTAACCA-3′ as primers and analyzed using ABI3730 capillary sequencer.

Statistical analysis

SNPAlyze software (DYNACOM, Japan) was used for the case–control study (χ^2-test), calculation of odds ratio and its 95% CI (Bootstrap method), haplotype analysis (Expectation–Maximization algorithm) and pairwise LD analysis (Lewontin's coefficient D' and standardized coefficient r).

Real-time RT–PCR

Autopsied frontal cortices were obtained from the Brain Bank for Aging Research (Tokyo Metropolitan Geriatric Hospital/Tokyo Metropolitan Institute of Gerontology) and from the Department of Neurology, Juntendo University School of Medicine. The samples contained 21 cases [age, 82.6 ± 7.1 (SD) years; 11 males and 10 females] with Lewy body pathology defined by the third Consensus Guideline for Dementia with Lewy Bodies (44), comprising PD with and without dementia and dementia with Lewy bodies, and 18 control subjects (age, 81.2 ± 5.2; 12 males and six females) without parkinsonism or dementia and without neurodegenerative pathological changes. Total RNA was extracted from tissues using RNeasy (Qiagen), and cDNA was prepared using Superscript reverse transcriptase (Invitrogen). Real-time RT–PCR was carried out on ABI PRISM 7900 sequence detection system (Applied Biosystems) using SYBR Premix Ex Taq (TAKARA, Japan). First-strand cDNA was amplified using primers specific for *SNCA* (forward: 5′-GCAGAAGCA GCAGGAAAGAC-3′; reverse: 5′-CTGGGCTACTGCTGTC ACAC-3′; product size: 159 bp) and *NF* (*neurofilament L*, forward: 5′-AGAACGCTGAGGAATGGTTC-3′; reverse: 5′-CTGGTGAAACTGAGTCGGGT-3′; product size: 391 bp). A single band of the expected size was amplified from cDNA samples, but not from RNA samples. For quantification, we used a relative standard curve method. Standard curves of *SNCA* and *NF* were generated from the amplification of diluted series of cDNA from cortices. *SNCA* expression levels were normalized to those of *NF*. One of the experimental samples was used as the calibrator. Each of the normalized *SNCA* values was divided by the calibrator normalized *SNCA* value to generate the relative expression levels. The values were determined in triplicate. Reproducibility of the results was confirmed by repeating cDNA synthesis and real-time PCR twice for seven samples, and similar results were obtained.

SUPPLEMENTARY MATERIAL

Supplementary Material is available at HMG Online.

ACKNOWLEDGEMENTS

We thank PD patients for participating in the study. We also thank Dr Ryo Yamada for graphical LD analysis; Drs Jun Ohashi, Toshihiro Tanaka and Shiro Ikegawa for helpful comments; Mio Yoshida, Drs Helena A. Popiel, Yushi Hirota and Katsushi Tokunaga for help of performing the study and Dr Jennifer Logan for editing the manuscript. This work was supported by the 21st Century COE program and Research Grant (14013037 and 17590874), both from the Ministry of Education, Culture, Sports, Science and Technology of Japan; by the Research Grant for Nervous and Mental Disorders (14B-3) and Grant for Research on Measures for Intractable Diseases (H14-Q-15-1), both from the Ministry of Health, Labor and Welfare of Japan and by a grant from Core Research for Evolutional Science and Technology (CREST) of the Japan Science and Technology Agency (JST).

Conflict of Interest statement. None declared.

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