Clinical evaluation, drug treatment regimen, and blood sampling

We performed routine laboratory tests including blood cell counts, liver and renal function tests, urinalysis, electrolyte and blood sugar measurements, and thyroid function tests during the first visit. The subjects were initially administered PAX (10 mg/day) at bedtime for 2 weeks as treatment for PD. Subjects with insomnia were prescribed brotizolam (0.25 or 0.5 mg) at bedtime (n=9), and those who had frequent panic attacks (n=15) were permitted to take a low dose of lorazepam ($\leq$ 2.0 mg/day). PD severity was assessed using the PAS observer-rated version [22] at baseline and 2 weeks after the initiation of drug treatment. PAS has the advantage of being able to assess different aspects of PD separately using the five subscores of the scale (A: panic attacks, B: agoraphobic avoidance, C: anticipatory anxiety, D: disability, E: worries about health).

Patients were maintained on PAX for 2 weeks, and 7 ml of venous blood was collected 10–15 h after the last evening dose into Venoject tubes with EDTA-Na (Terumo Japan, Tokyo, Japan). Blood samples were centrifuged at 3,000 g for 10 min, and aliquots of the plasma and cell fraction were separated, frozen, and stored at −80°C until analysis.

Selection of subjects

Out of the 38 enrolled subjects, 8 showed plasma concentrations under the limit of detection, indicating poor compliance. These eight subjects were excluded from the analysis because the accurate value of the plasma concentration of PAX could not be determined since the inter- and intra-assay coefficients of variation (CVs) would be more than 20%, and accuracy is not assured below the limit of detection. There is a possibility that a very low concentration of PAX is observed in the subjects with gene duplication of *CYP2D6* [23, 24] because PAX is one of the representative substrates of CYP2D6; however, this is unlikely because the frequency of gene duplication of *CYP2D6* is very low in Japanese [25].

As shown in Table 1, a total of five subjects had adverse effects; two dropped out because of these effects, namely, daytime drowsiness (female, 25 years old, S/S genotype) and abnormal sensation (male, 39 years old, L/S genotype). Additionally, one subject refused blood collection just before sampling. Thus, a total of 11 subjects (8 subjects showed PAX concentration under the low limit of detection; 1 subject refused blood sampling; 2 subjects dropped out due to adverse effects) were excluded from the analysis. Accordingly, data from the remaining 27 subjects (male=6, female=21) were analyzed in the present study; their demographic data are shown in Table 2.

Table 1 Characteristics of subjects with adverse effects

Gender	Age	Adverse effect	5-HTTLPR genotype
Female	25	Daytime drowsiness	S/S
Female	31	Daytime drowsiness	L/S
Female	32	Daytime drowsiness	L/S
Female	46	Daytime drowsiness	S/S
Male	39	Abnormal sensation	L/S

Determination of plasma concentration of PAX

The plasma concentration of PAX was measured by column-switching high-performance liquid chromatography (HPLC) with ultraviolet detection, as developed by Hikida et al. [26]. Drugs in the plasma, to which cisapride had been added as an internal standard, were extracted with hexane-chloroform. The extract was subjected to an automated column-switching HPLC using a hydrophilic meta-acrylate polymer column for sample clean-up and a reversed-phase column for separation. The lowest limit of detection was 0.5 ng/ml, and the interassay CV was < 5% at 1 ng/ml PAX. The data on the plasma concentration of PAX have partly been analyzed and published in our previous report [13].

Genotyping

Genomic DNA was isolated from the blood-cell fraction using the QIAamp Blood kit (QIAGEN, Chatsworth, CA, USA). 5-HTTLPR genotypes (L and S alleles) were determined by polymerase chain reaction (PCR) techniques described by Lesch [15, 16] and Heilis et al. [16] with minor modification. Oligonucleotide primers flanking the 5-HTTLPR and corresponding to the nucleotide positions ranging from −1416 to −1397 (LPR5; 5'-GGCGTTGCC GCTCTGAATTGC) and from −910 to −889 (LPR3; 5'-GAGGGACTGAGCTGGACAACCCAC) of the 5-HTT gene regulatory region were used to generate a 484/528-bp fragment. PCR amplification was carried out in a final volume of 12.5 µl consisting of 20 ng of genomic DNA, 0.8 mM dNTP mixture, 0.05 µg of sense and antisense primers (i.e., LPR5 and LPR3), 1×PCR buffer, 1.5 mM $MgCl_2$, 5% dimethyl sulfoxide, and 0.5 U of AmpliTaq DNA polymerase (Applied Biosystems Japan, Tokyo, Japan). Annealing was performed at 60°C for 30 s, extension at 72°C for 1 min, and denaturation at 94°C for 30 s for 35 cycles.

Statistical analysis

For statistical analysis we used linear regression analysis, multiple regression analysis, and Fischer's exact probability test using SPSS version 12.0 (SPSS Japan, Tokyo, Japan)

Table 2 Demographic characteristics at baseline according to 5-HTTLPR genotypes

5-HTTLPR genotypes	L/S	S/S	Total
Number of patients	9	18	27
Male/female	1/8	5/13	6/21
Age (years)	34.4±7.6	34.9±12.7	34.7±11.1
Body weight (kg)	56.0±9.8	54.4±8.4	54.9±8.7
With major depressive disorder	3	2	5
With/without agoraphobia	6/3	15/3	21/6
Panic attacks/week	1.8±1.3	2.7±4.3	2.4±3.6
Panic and agoraphobia scale	21.7±6.7	23.0±6.1	22.6±6.2

Values are presented as number or mean ± SD

and Prism version 2.0 (GraphPad, San Diego, CA, USA). P values < 0.05 were considered significant.

Results

The mean PAS score was 21.6±6.9 (range, 9–34) at baseline before the initiation of pharmacotherapy with PAX. After 2 weeks of treatment, the mean PAS score improved to 15.1±6.9 (2–31). The mean percent reduction in PAS score (%) [percent reduction in PAS score = ([PAS score at baseline – PAS score 2 weeks after the initiation of PAX treatment)/PAS score at baseline) × 100) was 31.4± 27.9% (−9.1 to 86.2%).

Figure 1 shows the relationship between the percent reduction in PAS score and the plasma concentration of PAX 2 weeks after the initiation of PAX administration. There was an approximately 23-fold interindividual variation in the plasma concentration of PAX (2.6–59.3 ng/ml) and also a large interindividual variation in the percent reduction in PAS score (−9.1 to 86.2%). There was a significant negative correlation between the percent reduction in PAS score and the plasma concentration of PAX irrespective of genotypes (R=0.42, P= 0.02). Moreover, there was a significant negative correlation between the percent reduction in PAS score and the plasma concentration of PAX in subjects with the S/S genotype of 5-HTTLPR (R=0.61, P=0.006), while no significant correlation between the percent reduction in PAS score and the plasma concentration of PAX in those with the L/S genotype of 5-HTTLPR was observed (R= 0.33, P=0.37).

As stated in the "Introduction" section, a PAX concentration of 28 ng/ml is suggested to be the upper end of the optimal range of PAX concentration from a PET study [14]. As shown in Fig. 1, there were no subjects with PAX concentration > 28 ng/ml showing a percent reduction of PAS score > 20%. In the PAX concentration range under 28 ng/ml, the subjects (92.9%) with the S/S genotype were more likely to show a percent reduction in PAS score > 20% than those with the L/S genotype [see Fig. 1; 13 out of 14 subjects (92.9%) vs 4 out of 9 subjects (44.4%), Fisher's exact probability test, P=0.018].

Multiple regression analysis was performed in order to analyze the relationship between demographic variables from the subjects and the clinical response to PAX (percent reduction in PAS score). We used the plasma concentration of PAX, age, gender, body weight, comorbid physical illness, comorbid major depressive disorder, comorbid agoraphobia, smoking status, habitual use of alcohol, PAS score at baseline, frequency of panic attacks per week at baseline, 5-HTTLPR genotype (L/S or S/S), adverse effect of PAX, use of lorazepam and/or brotizolam as independent variables and the clinical response to PAX (percent reduction in PAS score) as the dependent variable. Plasma concentration of PAX, 5-HTTLPR genotype, and comorbid physical illness were

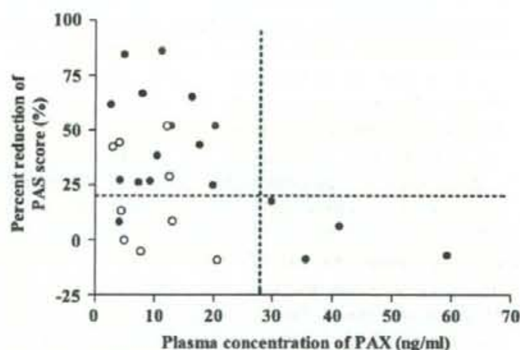

Fig. 1 Relationship between percent reduction in PAS score (*ordinate*), plasma concentration of PAX (*abscissa*) and 5-HTTLPR genotype (*empty circles* = subjects with the L/S genotype; *filled circles* = subjects with the S/S genotype) 2 weeks after the initiation of PAX administration. *Horizontal* and *vertical dashed lines* indicate 20% reduction in PAS score and PAX plasma concentration of 28 ng/ml, respectively. Note that a negative correlation between the percent reduction in PAS score and the plasma concentration of PAX was observed, and no subjects with a PAX concentration > 28 ng/ml showed a percent reduction in PAS score > 20%. In the PAX concentration range under 28 ng/ml, 13 out of 14 subjects (92.9%) with the S/S genotype showed a percent reduction in PAS score > 20%, while only 4 out of 9 subjects (44.4%) with the L/S genotype showed a percent reduction in PAS score > 20% (Fisher's exact test, P=0.018)

found to be significant factors affecting the percent reduction in PAS score (see Table 3); the correlation coefficient (R) for the full model was 0.724, indicating that these factors accounted for 52.4% (R^2=0.524) of the variability in the clinical response to PAX. The final model was thus described by the following equation (P=0.001): percent reduction in PAS score (%) = 68.5 − 1.2 × [plasma concentration of PAX (ng/ml)] − 33.0 × (L/S=1, S/S=0) − 21.8 × (with comorbid physical illness=1, without comorbid physical illness=0) (see Table 3).

Discussion

Stahl reported that PD patients tend to be more sensitive to SSRIs than depressed patients, since they can easily develop short-term worsening of their symptoms when pharmacotherapy is initiated [27]. Thus, PD patients are usually started at a lower dose than depressed patients [27]. Louie et al. reported that PD patients with accompanying major depressive disorder showed lower tolerability to SSRIs than patients with major depressive disorder alone [28]. Gilles et al. reported different upper thresholds of serum concentrations along the progression of pharmacotherapy in major depression, and they speculated that a high serum concentration of SSRIs may lead to 5-HTergic side effects such as anxiety or disturbances in sleep, appetite, and sexual function, which may impede the overall response to pharmacotherapy with SSRIs [29].

Table 3 Results of stepwise multiple regression analysis

Independent variable	P
Gender	0.386
Age	0.350
Body weight	0.428
Comorbid major depressive disorder	0.405
With/without agoraphobia	0.618
Smoking status	0.478
Habitual use of alcohol	0.929
PAS score at baseline	0.216
Initial panic attacks per week at baseline	0.965
Use of lorazepam and/or brotizolam	0.761
Adverse effect	0.603
Plasma concentration of paroxetine	0.001
5-HTTLPR genotype	0.001
Comorbid physical illness	0.016

The final model was described by the following equation (P=0.001): percent reduction in PAS score (%) = 68.5 − 1.2 × [plasma concentration of PAX (ng/ml)] − 33.0 × (L/S=1, S/S=0) − 21.8 × (with comorbid physical illness=1, without comorbid physical illness=0) [R=0.724, P=0.001, coefficient of determination (R^2) = 0.524]

In the present study, the plasma concentration of PAX has been shown to be one of the important determinants of the initial clinical response and a high plasma concentration of PAX has been shown to impede such a clinical response. Why does a high plasma concentration of PAX impede a favorable clinical response at the initial phase of pharmacotherapy (2 weeks) in PD? The cell bodies of 5-HT neurons are located in the brainstem area, that is, the raphe nucleus, and projections from the raphe nucleus to the frontal cortex are thought to be important for regulating mood. Projections from the raphe nucleus to the amygdala and prefrontal cortex may also play an important role in inhibiting anxiety. 5-HTergic terminals from the raphe nucleus to the prefrontal cortex inhibit emotional input from the prefrontal cortex to the amygdala [30]. 5-HTergic terminals from the raphe nucleus to the amygdala act as brakes on outputs from the amygdala to a fear response [30]. Thus, treatment with SSRIs has dual processes in that it diminishes the precipitation of both anxiety and fear [30]. SSRIs inhibit 5-HTT, leading to an increase in the amount of 5-HT in the synaptic cleft. The increase in 5-HT in the somatodendritic area of the 5-HT neurons causes desensitization or down-regulation of somatodendritic 5-HT_{1A} autoreceptors, and following the down-regulation of these autoreceptors, the 5-HT neuronal impulse flow is increased. This inhibits both the emotional input from the prefrontal cortex to the amygdala and the output from the amygdala to a fear response.

However, at the initial phase of pharmacotherapy with SSRIs, particularly before the down-regulation of 5-HT_{1A} autoreceptors, the amount of 5-HT increases to a higher level at the cell body area in the raphe nucleus than in the axon terminals [30]. 5-HT_{1A} receptors, which are autoreceptors located on cell bodies and dendrites, detect the increase in 5-HT and cause a shutdown of the 5-HT neuronal impulse flow before the desensitization, which might lower the activity of the 5-HT projection to both the prefrontal cortex and the amygdala from the raphe nucleus.

Accordingly, the increase in 5-HT in the somatodendritic area is indispensable for desensitizing the 5-HT_{1A} receptors and for inhibiting PD symptoms; however, the increase in 5-HT in the somatodendritic area before the down-regulation of 5-HT_{1A} receptors may lead to the deterioration of PD symptoms, which might be caused by a high plasma concentration of PAX.

Perna et al. reported that both the L/L genotype and the L/S genotype of 5-HTTLPR showed a significantly better response to PAX than the S/S genotype in female PD patients 12 weeks after the initiation of pharmacotherapy [21]. In contrast to the report of Perna et al. [21], the patients with the L/S genotype of 5-HTTLPR showed a lower clinical response than those with the S/S genotype in the present study. The difference in the results between the

two studies might be due to the difference in the observation duration, i.e., 2 weeks in the present study vs. 12 weeks in the study of Perna et al. [21].

Stahl reported that SSRIs cause a shutdown of the 5-HT neuronal impulse flow before the desensitization of 5-HT_{1A} autoreceptors, which might lead to the decline of PD symptoms [27]. Consequently, L allele carriers were assumed to be more sensitive to the pharmacological effects of PAX than those with the S/S homozygote.

In the present study, the therapeutic response of the subjects with the S/S genotype to PAX was better than that of the subjects with the L/S genotype 2 weeks after the initiation of pharmacotherapy with PAX. In order to explain the present finding on the 5-HTTLPR genotypes, we must speculate about the status of 5-HT_{1A} receptors, which are autoreceptors located on cell bodies and dendrites, in drug naïve patients.

David et al. used ^{11}C-*N*-(2-(4-(2-methoxyphenyl)-1-piperazinyl)ethyl)-*N*(2-pyridyl)cyclohexanecarboxamide ([^{11}C]-WAY) as a selective radioligand for the 5-HT_{1A} receptor and reported that the 5-HT_{1A} receptor genotype showed no significant effects on 5-HT_{1A} receptor binding in a PET study. On the other hand, 5-HT_{1A} receptor binding potential values were lower in all brain regions including the raphe nucleus in healthy subjects with the S/S or L/S genotype of 5-HTTLPR than in healthy individuals with the L/L genotype [31]. When the same process occurred in the drug-naïve patients with PD, the drug-naïve patients with the S/S genotype of 5-HTTLPR showed decreased 5-HTT function, and this condition might lead to a lifelong increase in the concentration of 5-HT in the synaptic cleft. Additionally, the 5-HT_{1A} receptor in the drug-naïve patients with the S/S genotype of 5-HTTLPR is thought to be, as it was proven, "down-regulated" compared with the drug-naïve patients with the L/S genotype.

In the present study, comorbid physical illness was shown to be associated with a poor response to PAX. The presence of medical comorbidity complicates the identification, presentation, and treatment of PD because a number of physical illness including cardiovascular disease, respiratory disorders, and vestibular and thyroid dysfunctions have symptoms that overlap with symptoms of PD [32].

As shown in Table 1, two subjects with the S/S genotype and three subjects with the L/S genotype reported adverse effects. Understanding the relationship between the 5-HTTLPR genotype and the development of adverse effects has been controversial. Murphy et al. reported that S allele carriers experienced more severe adverse events during pharmacotherapy with PAX [33]. Perlis et al. reported that the S allele may be used to identify patients at risk for developing insomnia or agitation with fluoxetine treatment [34]. In contrast, Takahashi et al. reported no association between 5-HTTLPR and the development of nausea during treatment with fluvoxamine [35]. Moreover, Kato et al. reported no association between 5-HTTLPR and adverse effects during treatment with fluvoxamine and PAX [36].

The present study has several limitations. Firstly, only one genetic polymorphism, i.e., 5-HTTLPR, was assessed. PD is considered to be a polygenic disorder, and it is believed that other genes such as 5-HT receptor genes and progesterone receptor genes might contribute to the pathogenesis of PD. Secondly, this study analyzed a relatively small sample size due to the high drop-out rate. This resulted in having no subjects with the L/L genotype, because the frequency of the L/L genotype in Japanese has been reported to be approximately only 3% [37]. Thirdly, the present analysis has been limited to only the early phase of the pharmacotherapy for PD, i.e., 2 weeks after the initiation of pharmacotherapy. Since PD is a chronic illness, it would also be important to determine the clinical response in the later phase of pharmacotherapy. Accumulation of data on the clinical response of PD to PAX in the later phase of pharmacotherapy is in progress.

In conclusion, high plasma concentration of PAX, 5-HTTLPR L/S genotype, and comorbid physical illness are associated with a poor response of PD to PAX in the initial phase of pharmacotherapy.

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Replication Study and Meta-Analysis of the Genetic Association of GRM3 Gene Polymorphisms With Schizophrenia in a Large Japanese Case-Control Population

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The GRM3 gene, which encodes a metabotropic glutamate receptor, is an important candidate gene for susceptibility to schizophrenia. Two single nucleotide polymorphisms (SNPs), rs1468412 and rs2299225 in intron 3, were reported to be associated with schizophrenia in Japanese and Chinese populations, respectively. Haplotypes with these SNPs were also reported to be associated with schizophrenia. In the present study, we attempted to replicate these single marker and haplotype associations in a case-control study of 1,916 Japanese patients with schizophrenia and 1,915 Japanese control subjects. In addition to these two SNPs, we genotyped rs274622 in the promoter region of GRM3. In the present study, none of these polymorphisms were associated with schizophrenia (rs274622, allelic $P = 0.68$; rs1468412, allelic $P = 0.74$; rs2299225, allelic $P = 0.20$). Haplotypes constructed with these SNPs also were not associated with schizophrenia ($P = 0.18$–0.84). Meta-analysis of five case-control studies of more than 3,000 patients with schizophrenia and more than 3,000 control subjects did not support the associations of rs1468412 and rs2299225 with schizophrenia. Our data indicate that SNPs previously reported to be associated with schizophrenia do not contribute to genetic susceptibility to schizophrenia.
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KEY WORDS: **glutamate receptor; single nucleotide polymorphism; haplotype**

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INTRODUCTION

The metabotropic glutamate receptors (mGluRs) modulate glutamate neurotransmission in the central and peripheral nervous systems. mGluRs mediate signal transduction through G-protein second messenger systems [Pin and Duvoisin, 1995]. There are eight known genes (GRM1-GRM8) that encode mGluRs, and the receptors are subdivided into three groups (I–III) based on sequence homology, signal transduction, and pharmacological properties [Nakanishi, 1992]. The glutamatergic neurotransmitter system is involved in schizophrenia. Agents that directly or indirectly modulate the mGluRs may be potential drugs for treatment of schizophrenia [Holden, 2001]. The group II mGluR agonists have been shown to ameliorate the adverse behavioral effects induced by phencyclidine in mice [Moghaddam and Adams, 1998]. The group II receptors comprise mGluR2 and mGluR3, and therefore, GRM3 is an important candidate gene for susceptibility to schizophrenia.

Seven studies have reported genetic associations between GRM3 variations and schizophrenia; however, the results are inconsistent. While three studies found associations between single nucleotide polymorphisms (SNPs) in GRM3 and schizophrenia [Fujii et al., 2003; Egan et al., 2004; Chen et al., 2005], the remaining four did not [Marti et al., 2002; Fallin et al., 2005; Norton et al., 2005; Tochigi et al., 2006]. Fujii et al. [2003] reported that the A allele of the rs1468412 SNP in intron 3 of GRM3 was more common in 100 Japanese schizophrenia patients than in 100 Japanese control subjects (allelic $P = 0.01$;

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TABLE I. Genotypic and Allelic Distributions of Three SNPs of the *GRM3* Gene

Polymorphism	Area[a]	Group		Genotype count (frequency)			P	Allele count (frequency)		P	HWE P
rs274622				CC	CT	TT		C	T		
	WJ	Affected	n = 475	13 (0.03)	150 (0.32)	312 (0.66)		176 (0.19)	774 (0.81)		0.315
	WJ	Controls	n = 477	14 (0.03)	152 (0.32)	311 (0.65)	0.85	180 (0.19)	774 (0.81)	0.85	0.373
	EJ	Affected	n = 1439	37 (0.03)	403 (0.28)	999 (0.69)		477 (0.17)	2401 (0.83)		0.630
	EJ	Controls	n = 1438	35 (0.02)	390 (0.27)	1013 (0.70)	0.55	460 (0.16)	2416 (0.84)	0.55	0.726
	Total	Affected	n = 1914	50 (0.03)	553 (0.29)	1311 (0.68)		653 (0.17)	3175 (0.83)		0.357
		Controls	n = 1915	49 (0.03)	542 (0.28)	1324 (0.69)	0.68	640 (0.17)	3190 (0.83)	0.68	0.463
rs1468412				AA	AT	TT		A	T		
	WJ	Affected	n = 479	319 (0.67)	142 (0.30)	18 (0.04)		780 (0.81)	178 (0.19)		0.658
	WJ	Controls	n = 477	293 (0.61)	163 (0.34)	21 (0.04)	0.11	749 (0.79)	205 (0.21)	0.11	0.781
	EJ	Affected	n = 1436	924 (0.64)	457 (0.32)	55 (0.04)		2305 (0.80)	567 (0.20)		0.872
	EJ	Controls	n = 1432	935 (0.65)	445 (0.31)	52 (0.04)	0.58	2315 (0.81)	549 (0.19)	0.58	0.916
	Total	Affected	n = 1915	1243 (0.65)	599 (0.31)	73 (0.04)		3085 (0.81)	745 (0.19)		0.937
		Controls	n = 1909	1228 (0.64)	608 (0.32)	73 (0.04)	0.74	3064 (0.80)	754 (0.20)	0.74	0.834
rs2299225				GG	GT	TT		G	T		
	WJ	Affected	n = 479	8 (0.02)	83 (0.17)	388 (0.81)		99 (0.10)	859 (0.90)		0.155
	WJ	Controls	n = 477	10 (0.02)	105 (0.22)	362 (0.76)	0.07	125 (0.13)	829 (0.87)	0.06	0.466
	EJ	Affected	n = 1437	18 (0.01)	309 (0.22)	1110 (0.77)		345 (0.12)	2529 (0.88)		0.499
	EJ	Controls	n = 1433	22 (0.02)	310 (0.22)	1101 (0.77)	0.69	354 (0.12)	2512 (0.88)	0.69	0.973
	Total	Affected	n = 1916	26 (0.01)	392 (0.20)	1498 (0.78)		444 (0.12)	3388 (0.88)		0.951
		Controls	n = 1910	32 (0.02)	415 (0.22)	1463 (0.77)	0.20	479 (0.13)	3341 (0.87)	0.20	0.681

[a]Area where the subjects lived, WJ: western Japan (Okayama area); EJ: central to eastern Japan (Kanto, Niigata, Nagoya area) HWE: Hardy–Weinberg Equilibrium.

TABLE II. Estimated Haplotype Frequencies of the *GRM3* Gene

Haplotype	Schizophrenia frequency	Control frequency	*P*
rs274622-rs1468412-rs2299225			
T-A-T	0.65	0.65	0.81
C-A-T	0.16	0.15	0.59
T-T-G	0.11	0.12	0.23
T-T-T	0.07	0.07	0.19
rs274622-rs1468412			
T-A	0.65	0.65	0.81
T-T	0.18	0.18	0.93
C-A	0.16	0.15	0.52
C-T	0.01	0.01	0.44
rs1468412-rs2299225			
A-T	0.80	0.80	0.84
T-G	0.11	0.12	0.18
T-T	0.08	0.07	0.21

odds ratio = [OR] 1.92; 95% confidence interval [CI] = 1.18–3.12). Chen et al. [2005] reported that the C allele of the rs2299225 SNP in intron 3 of GRM3 was found more frequently in 752 Chinese patients with schizophrenia than in 752 control subjects ($P = 0.03$; OR = 1.44; 95% CI = 1.05–1.99). Egan et al. [2004] reported that the A allele of the rs6465084 SNP in intron 2 was overtransmitted to affected offspring in European-American families included in the Clinical Brain Disorders Branch "Sibling Study" ($P = 0.02$); however, this allele was undertransmitted to affected offspring in Caucasian families, who participated in the National Institute of Mental Health Genetics Initiative (NIMHGI; $P = 0.27$). These three papers also reported haplotype associations with schizophrenia. However, the SNPs used to construct haplotypes differed between the studies. Marti et al. [2002] reported that the T allele of rs2228595, a synonymous SNP in exon 3, was overrepresented in 265 German schizophrenia patients compared with that of 227 control subjects ($P = 0.002$). However, this association was not replicated in another study of 289 German patients and 163 control subjects ($P = 0.57$). Fallin et al. [2005] reported significant associations of 4-SNP haplotypes of GRM3 with bipolar disorder but not with schizophrenia in Ashkenazi Jewish case-parent trios. Tochigi et al. [2006] did not observe associations between 10 SNPs in GRM3 and schizophrenia in 402 Japanese patients with schizophrenia and 468 Japanese control subjects. Thus, the findings that support associations of genetic variations in GRM3 with schizophrenia are not compelling, although GRM3 is regarded as one of the most promising candidate genes [Harrison and Weinberger, 2005].

Genetic association studies are prone to spurious findings and type 1 errors, and therefore, replication studies are essential before genetic association is accepted. However, in replication studies, the sample size is critical because the association of SNPs with schizophrenia is thought to be weak. Studies without sufficient power to replicate a previously reported association are prone to type 2 errors. In the present study, we attempted to confirm associations of two SNPs in GRM3 reported to be associated with schizophrenia in Asian populations [Fujii et al., 2003; Chen et al., 2005]. In addition to these two SNPs, we genotyped one SNP, rs274622, that was reported to be associated with negative symptom improvement in Caucasian schizophrenia patients treated with olanzapine [Bishop et al., 2005].

MATERIALS AND METHODS

Subjects

All subjects were of Japanese descent and were recruited from the main island of Japan. A total of 1,916 unrelated patients with schizophrenia (mean age ± SD, 48.9 ± 14.5 years; 1058 men and 858 women) were diagnosed according to the Diagnostic and Statistical Manual of Mental Disorders, Fourth Edition (DSM-IV). Control subjects were 1,915 mentally healthy, unrelated subjects (mean age ± SD, 49.0 ± 14.3 years; 1,044 mens and 871 womens) with no self-reported family history of mental illness within second-degree relatives. The present study was approved by the Ethics Committees of the University of Tsukuba, Niigata University, Fujita Health University, Nagoya University, Okayama University, and Teikyo University. All participants provided written informed consent.

Genotyping

DNA was extracted from blood samples. We genotyped three SNP markers, rs274622 in the promoter region, and rs1468412 and rs2299225 in intron 3 of GRM3. The SNPs were genotyped by TaqMan assay. Predesigned TaqMan SNP genotyping assays, C_2293486_10 for rs274622, C_15752033_10 for rs1468412, and C_7586401_10 for rs2299225, were selected from the Applied Biosystems database (http://www.appliedbiosystems.com). The TaqMan reaction was performed in a final volume of 3 µl consisting of 2.5 ng genomic DNA and Universal Master Mix (Eurogentic, Seraing, Belgium). Genotyping was performed with an ABI PRISM 7900HT Sequence Detection System (Applied Biosystems, Foster City, CA).

Statistical Analysis

Hardy–Weinberg equilibrium, linkage disequilibrium, and allelic/haplotype frequencies, as well as an association between an SNP or haplotype and schizophrenia, were evaluated with the Haploview software program (http://www.broad.mit.edu/mpg/haploview/). Permutation tests were also performed to calculate corrected *P* values for multiple testing with the Haploview software. Genotype-based association was tested with Cochran–Armitage test for trends. Statistical significance was accepted at $P < 0.05$.

Meta-analysis was performed with the Mantel–Haenszel method as a fixed-effect model test and the DerSimonian–Laird method as a random-effects model test. Heterogeneity among studies was tested with the χ^2 statistic obtained by adding the weighted squares of the deviation of each estimate from the pooled estimate. Publication bias was not examined because the number of reports was small. We estimated ORs by comparing patients with schizophrenia with control subjects in the same study and calculated ORs under the hypothesis that the A and G alleles of rs1468412 and rs2299225, respectively, were more susceptible to schizophrenia as shown in previous studies [Fujii et al., 2003; Chen et al., 2005].

TABLE III. Meta-Analysis of Five Case-Control Studies of GRM3 Polymorphisms and Schizophrenia

References	Population	The A allele of rs1468412: Patients with schizophrenia (n)	Control (n)	OR	(95% CI)	P	The G allele of rs2299225: Patients with schizophrenia (n)	Control (n)	OR	(95% CI)	P
Fujii et al. (2003)	Japanese	100	100	1.92	(1.18–3.12)	0.01					
Chen et al. (2005)	Chinese	735	733	0.95	(0.75–1.20)	0.22	751	750	1.44	(1.05–1.99)	0.03
Norton et al. (2005)	German	663	698	0.93	(0.79–1.10)	0.41					
Tochigi et al. (2006)	Japanese	402	457	0.89	(0.74–1.07)	0.65	401	463	1.20	(0.89–1.63)	0.23
Present Study	Japanese	1915	1909	1.02	(0.91–1.14)	0.74	1916	1910	0.91	(0.80–1.05)	0.2
Combined fixed-effects model (Asian)				1.00	(0.92–1.09)	1.00			1.01	(0.91–1.13)	0.81
Combined random-effects model (Asian)		3152	3199	1.03	(0.91–1.14)	0.61	3068	3123	1.13	(0.85–1.51)	0.52
Combined fixed-effects model (total)				0.99	(0.92–1.06)	0.87					
Combined random-effects model (total)		3815	3897	1.00	(0.87–1.14)	1.00					

RESULTS

Genotypic/allelic distributions of the three GRM3 SNPs among the subject groups are shown in Table I. Distributions of these SNPs did not differ significantly from Hardy–Weinberg equilibrium. No significant genotype/allelic association with schizophrenia was detected for rs274622 (genotypic $P = 0.68$/allelic $P = 0.68$), rs1468412 (genotypic $P = 0.74$/allelic $P = 0.74$), or rs2299225 (genotypic $P = 0.20$/allelic $P = 0.20$). Populations in east and west Japan have slightly different cultural backgrounds. Therefore, we divided our subjects into those who lived in eastern and western Japan. No significant association was observed (Table I). These SNPs were in linkage disequilibrium with each other in both the control and patient groups as reported by Tochigi et al. [2006] ($D' = 0.60$ and $r^2 = 0.02$ between rs274622 and rs1468412; $D' = 0.98$ and $r^2 = 0.54$ between rs1468412 and rs2299225; $D' = 0.78$ and $r^2 = 0.02$ between rs274622 and rs2299225, respectively, in the total subjects). Haplotype frequencies did not differ significantly between the schizophrenia and control groups (Table II).

The total number of subjects included in the meta-analysis was 3,815 patients with schizophrenia and 3,897 control subjects for rs1468412 and 3,068 patients and 3,123 control subjects for rs2299225 (Table III). Overall, the combined OR of the fixed-effects model for schizophrenia with rs1468412 was 0.99 (95% CI = 0.92–1.06; $P = 0.87$; heterogeneity $P = 0.06$), and that of rs2299225 was 1.01 (95% CI = 0.91–1.05; $P = 0.67$; heterogeneity $P = 0.02$). Meta-analysis limited to Asian studies also yielded no significant results (Table III).

DISCUSSION

We attempted to replicate genetic associations between rs1468412 and rs2299225 that were previously reported to be associated with schizophrenia in a Japanese population [Fujii et al., 2003] and a Chinese population [Chen et al., 2005], respectively. We were not successful in replicating either association. As shown in Table III, Fujii et al. [2003] reported the OR for schizophrenia with the A allele of rs1468412 was 1.92 (95% CI = 1.18–3.12), whereas, in the present study, the OR was 1.02 (95% CI = 0.91–1.14). The power of detection for the OR of 1.18 (the lower end of the 95% CI for OR reported by Fujii et al. [2003]), was >0.9 in the present study. Moreover, the result of the meta-analysis did not support the association of rs1468412 with schizophrenia. The meta-analysis of the present study did not include transmission disequilibrium test (TDT) data for rs1468412 which were reported by Egan et al. [2004]. However, their study did not detect a significant association of the SNP with schizophrenia.

Chen et al. [2005] reported that the OR for association of the G allele of rs2299225 with schizophrenia was 1.44 (95% CI = 1.05–1.99), whereas the OR in the present study was 0.91 (95% CI = 0.8–1.05). The 95% CIs of the ORs of the study reported by Chen et al. [2005] and the present study did not overlap. The meta-analysis in the present study did not support the association of rs2299225 with schizophrenia. Thus, the present study provides further evidence that neither SNP in GRM3 is associated with schizophrenia.

In the present study, significant haplotype associations with schizophrenia were not detected. Chen et al. [2005] reported that haplotype of C (rs2237562)—T (rs1468412)—C (rs2299225) was detected more frequently in patients with schizophrenia than in control subjects ($P = 0.008$). Although we did not genotype rs2237562, the rs1468412 and rs2299225 SNPs can determine the haplotype due to linkage disequilibrium. We observed no difference in frequencies of haplotypes constructed by rs1468412 and rs2299225 between patients with schizophrenia and controls in the present study (Table II). Fujii et al. [2003] reported significant associations of seven 2-SNP haplotypes and

five 3-SNP haplotypes, including the haplotype constructed with rs274622 and rs1468412 ($P = 0.002$), with schizophrenia. The present study, however, did not replicate the rs274622 and rs1468412 haplotype associations (Table II).

In the present study, we examined only three SNPs. Therefore, the present study does not exclude the possibility of associations between other variations in GRM3 and schizophrenia. Although the GRM3 single marker and haplotype associations with schizophrenia have not been replicated so far in other populations, further studies exploring associations of genetic variations not in linkage disequilibrium with the GRM3 SNPs or haplotypes examined thus far with schizophrenia are necessary. Furthermore, epistatic interactions between variations in GRM3 and those in other genes remain mostly to be investigated. A marginal significant interaction between polymorphisms in GRM3 and catechol-*O*-methyltransferase (COMT) Val158Met has been reported recently [Nicodemus et al., 2007].

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BIOLOGICAL PSYCHIATRY - ORIGINAL ARTICLE

Genetic association analysis of tagging SNPs in alpha4 and beta2 subunits of neuronal nicotinic acetylcholine receptor genes (*CHRNA4* and *CHRNB2*) with schizophrenia in the Japanese population

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Abstract Several lines of evidence suggest that nicotinic cholinergic dysfunction may contribute to the cognitive impairments in schizophrenia. The majority of high affinity nicotine binding sites in the human brain have been implicated in heteropentameric alpha4 and beta2 subunits of neuronal nicotinic acetylcholine receptors; therefore, these two neuronal nicotinic acetylcholine receptors genes (*CHRNA4* and *CHRNB2*) are considered to be attractive candidate genes for the pathophysiology of schizophrenia. To represent these two genes in a gene-wide manner, we first evaluated the linkage disequilibrium structure using our own control samples. Thirteen SNPs (7 SNPs for *CHRNA4* and 5 SNPs for *CHRNB2*) were selected as tagging SNPs. Using these tagging SNPs, we then conducted genetic association analysis of case-control samples (738 schizophrenia and 753 controls) in the Japanese population. No significant association was detected in the allele/genotype-wise or haplotype-wise analysis. Our results suggest that *CHRNA4* and *CHRNB2* do not play a major role in Japanese schizophrenia.

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Keywords Schizophrenia · *CHRNA4* · *CHRNB2* · Linkage disequilibrium · Tagging SNP

Introduction

Cognitive impairments in areas such as attention, executive function, language and memory, for which there is not much hope of recovery with treatment, have been implicated as endophenotypes for schizophrenia (Green 1996), and such impairments may be partially mediated by nicotinic acetylcholine receptors (Levin and Simon 1998). A recent study has reported an association of such cognitive impairments with abnormalities in the neuronal network in the prefrontal cortex, superior temporal gyrus and cerebellum in schizophrenics (Bonilha et al. 2008). The smoking rate of schizophrenics is much higher than that of healthy individuals with lifetime history (Hughes et al. 1986). Schizophrenics may smoke to compensate for their cognitive dysfunctions, since nicotine has cognitive-enhancing properties (Kumari and Postma 2005).

The majority of high affinity nicotine binding sites in the brain consist of heteropentameric alpha4 and beta2 subunits of neuronal nicotinic acetylcholine receptors (nAChRs) (Flores et al. 1997). Several lines of evidence support an association between abnormalities in alpha4 and beta2 subunits and schizophrenia. Firstly, beta2 subunits in the ventral tegmental area in mutant mice showed alternation of dopamine release in the nucleus accumbens and changes in cognitive functions such as navigation and exploratory behaviour compared with wild mice (Maskos et al. 2005). Alpha4 subunit mutant mice also showed increased anxiety-like behaviour and a reduction of substantia nigra dopaminergic neurons on ageing (Labarca et al. 2001; Ross et al. 2000). These animal study results

may reflect dopamine and neurodevelopmental hypotheses of the pathophysiology of schizophrenia, and these behaviors may be major symptoms of schizophrenia (Lang et al. 2007). Secondly, a postmortem study reported fewer alpha4/beta2 subunits in the hippocampi of schizophrenics (Freedman et al. 1995). Thirdly, De Luca et al. reported that the alpha4 gene (*CHRNA4*) and beta2 subunit gene (*CHRNB2*) showed genetic interactions with schizophrenia in a family-based association study in the Canadian population (De Luca et al. 2006), and *CHRNA4* and *CHRNB2* were also associated with smoking among schizophrenics (Faraone et al. 2004; Voineskos et al. 2007).

Therefore, we conducted a genetic association study of *CHRNA4* (located on 20q13) and *CHRNB2* (located on 1q21) with schizophrenia in the Japanese population. In this study, we applied the "gene-wide" approach recommended by Ikeda to consider the population differences (Ikeda et al. 2008), first evaluating the linkage disequilibrium (LD) structure of these genes and selecting tagging SNPs ("tag SNPs"). These tag SNPs were then used to present the LD properties of the gene in the Japanese population in the following association analysis.

Materials and methods

Subjects

The subjects in the association analysis were 738 schizophrenia patients (395 males and 343 females; mean age ± standard deviation (SD) 47.4 ± 16.6 years; age of onset 26.0 ± 9.55 years) and 753 healthy controls (326 males and 427 females; 37.3 ± 14.3 years). Patients were grouped according to the following DSM-IV subtypes of schizophrenia: Paranoid Type ($n = 216$), Disorganized Type ($n = 221$), Catatonic Type ($n = 29$), Residual Type ($n = 142$), Undifferentiated Type ($n = 130$). All healthy controls are identical to those in our previous paper (Kishi et al. 2008). The subjects for LD evaluation were 96 controls, who were also among the sample used in the association analysis. All subjects were unrelated to each other and ethnically Japanese. The patients were diagnosed according to DSM-IV criteria with the consensus of at least two experienced psychiatrists on the basis of unstructured interviews and a review of medical records. All healthy controls were also psychiatrically screened based on unstructured interviews that included current and past psychiatric history. None had serious medical complications such as cirrhosis, renal failure, heart failure or other Axis-I disorders according to DSM-IV. No structured methods were used to assess psychiatric symptoms in the controls that included hospital staff, their families and medical students.

After explaining the study to all subjects, written informed consent was obtained from each. This study was approved by the Ethics Committee at Fujita Health University and Nagoya University School of Medicine.

SNP selection and LD evaluation

Methods for selection of tagging SNPs were described in our previous paper (Kishi et al. 2008). Briefly, we first consulted the HapMap database (release#20/phaseII, Jan 2006, www.hapmap.org) to select the tag SNPs; however, no dense marker sets were listed in HapMap (2SNPs and 5SNPs in *CHRNA4* and *CHRNB2*, respectively). Therefore, we accessed another information source, the JSNP database (Haga et al. 2002; Hirakawa et al. 2002), and picked up 11 SNPs to evaluate the denser LD structure of *CHRNA4* (we tried to select SNPs with minor allele frequencies (MAFs) of more than 0.05 to increase the power).

At first, we genotyped all these SNPs using our own 96 control samples to evaluate the LD structure in the Japanese population. We then selected tag SNPs with the criteria of r^2 threshold greater than 0.8 in "pair-wise tagging only" mode using the "Tagger" program (Paul de Bakker, http://www/broad.mit.edu/mpg/tagger) in Haploview 3.2 for the following association analysis (Barrett et al. 2005; Gabriel et al. 2002).

SNP genotyping

For rapid genotyping of SNPs, we used TaqMan assays (Applied Biosystems) and direct sequencing (Table 1). Detailed information, including primer sequences and reaction conditions, is available on request.

Statistical analysis

The genotypic deviation from Hardy-Weinberg equilibrium (HWE) was evaluated by χ^2 test (SAS/Genetics, release 8.2, SAS Japan Inc, Tokyo, Japan).

Marker-trait association was evaluated by the χ^2 test (allele and genotype-wise analyses), and the log-likelihood test (haplotype-wise analysis; the haplotype frequencies were estimated with the expectation-maximization algorithm) (SAS/Genetics, release 8.2). In this haplotype-wise analysis, the information of the "LD block" (criteria based on 95% confidential intervals on the D' values) in the LD evaluation step were used.

Power calculation was performed using a statistical program prepared by Ohashi et al. (2001). The significance level for all statistical tests was 0.05.

Table 1 tag SNPs and association analysis of *CHRNA4* and *CHRNB2*

Gene symbol	SNP ID[a]	Phenotype	MAF	Genotype deviation				*P* value		
				N	M/M	M/m	m/m	HWE	Genotype	Allele
CHRNA4	SNP A-1 rs755203	SCZ	0.418	732	250	352	130	0.752	0.972	0.809
		CON	0.414	747	259	358	130	0.742		
	SNP A-2 rs2273506	SCZ	0.105	732	585	141	6	0.431	0.124	0.0542
		CON	0.127	747	564	176	7	0.0939		
	SNP A-6 rs2273504	SCZ	0.465	732	214	355	163	0.493	0.741	0.560
		CON	0.454	747	232	351	164	0.152		
	SNP A-9 rs1044396	SCZ	0.287	732	379	286	67	0.222	0.572	0.476
		CON	0.275	747	393	297	57	0.932		
	SNP A-10 rs1044397	SCZ	0.390	731	278	336	117	0.360	0.826	0.539
		CON	0.401	747	273	349	125	0.454		
	SNP A-12 rs2236196	SCZ	0.116	731	573	147	11	0.656	0.167	0.309
		CON	0.128	747	563	177	7	0.0874		
	SNP A-13 rs4522666	SCZ	0.422	732	240	366	126	0.501	0.567	0.650
		CON	0.430	747	247	357	143	0.490		
CHRNB2	SNP B-1 rs4845652	SCZ	0.116	735	579	141	15	0.0697	0.401	0.194
		CON	0.101	749	607	132	10	0.359		
	SNP B-2 rs2072658	SCZ	0.201	738	467	246	25	0.283	0.930	0.772
		CON	0.205	747	466	256	25	0.155		
	SNP B-3 rs2072659	SCZ	0.143	734	542	174	18	0.370	0.623	0.606
		CON	0.136	751	559	179	13	0.759		
	SNP B-4 rs2072660	SCZ	0.252	735	410	279	46	0.873	0.585	0.352
		CON	0.238	753	440	268	45	0.622		
	SNP B-5 rs3811450	SCZ	0.119	736	576	145	15	0.106	0.218	0.981
		CON	0.119	751	580	163	8	0.354		

P value for association of SNPs in *CHRNA4* and *CHRNB2* with schizophrenia

[a] tag SNPs

SCZ schizophrenia, *CON* controls, *MAF* minor allele frequency of 96 controls, *N* number, *M* major allele, *m* minor allele, *HWE* Hardy–Weinberg equilibrium

Results

For the initial LD evaluation, 13 SNPs (2SNPs from HapMap, 11SNPs from JSNP) and five SNPs (5SNPs from HapMap) for *CHRNA4* and *CHRNB2*, respectively, were genotyped for 96 controls. The LD structure results can be seen in our previous paper (Kishi et al. 2008). Seven and five SNPs were then selected as tag SNPs for each gene (Kishi et al. 2008). The genotypic distributions of all SNPs were in HWE.

No significant associations were found between any tag SNPs in *CHRNA4* and *CHRNB2* and Japanese schizophrenia in the allele/genotype-wise analysis (Table 1) or in the haplotype analysis (*CHRNA4* Block I-P: 0.502, Block II-P: 0.432, *CHRNB2* Block I-P: 0.564). To further investigate these associations, we included an explorative analysis of gender effects, because recent studies showed that genetic factors underlying nicotine addiction probably play a different role in female and male smokers (Feng et al. 2004; Greenbaum et al. 2006; Li et al., 2005). This suggests the existence of sex-specific genetic components in nicotine use disorders or these genes. In the present analysis, we found that SNP A-10 and B-4 were significantly associated with male and female schizophrenics, respectively (SNP A-10 and male schizophrenia P: 0.0389, SNP B-4 and female schizophrenia P: 0.0262). However, these results were no longer statistically significant after Bonferroni correction (SNP A-10 and male schizophrenia P: 0.545, SNP B-4 and female schizophrenia P: 0.262).

In the power analysis, we obtained power of more than 80% for the detection of association when we set the genotype relative risk at 1.23–1.36 and 1.26–1.35 for *CHRNA4* and *CHRNB2*, respectively, under a multiplicative model of inheritance.

Discussion

In this study, we found no significant association between two major cholinergic receptor genes, *CHRNA4* and *CHRNB2*, and schizophrenia in the Japanese population.

Although nominal significant associations between two SNPs (A-10 and B-4) and subgroups divided by gender were detected in the explorative analyses, these associations might have been the result of type I error due to multiple testing.

A recent family-based association study showed gene–gene interactions between *CHRNA4* and *CHRNB2* (De Luca et al. 2006). Therefore, we applied the recently recommended strategy of "gene-based" association analysis for the purpose of detecting susceptibility genes for schizophrenia (Neale and Sham 2004), and conducted a case-control association analysis by selecting the tag SNPs. As in the original study, we did not detect an association between these genes and schizophrenia. To evaluate the interactions with each SNP in these genes, we then analyzed the gene–gene interactions with the use of the Multifactor Dimensionality Reduction (MDR) method (Hahn et al. 2003). This analysis, however, revealed no interactions with schizophrenia (data not shown).

Voineskos et al. showed a significant association of rs3746372 in *CHRNA4* with heavy smoking in schizophrenia (Voineskos et al. 2007). The rs3746372 is located upstream 39593 bp from the initial exon and rs3746372. In this study, we selected tag SNPs in an association analysis after performing a LD evaluation that covered *CHRNA4*, including the promoter region, using our control samples. Although we confirmed LD between the SNPs selected in this study and rs3746372 according to the HapMap database, this LD was not found to be tight. Since we thought that rs3746372 might not be included in *CHRNA4*, we did not perform an association analysis of this SNP.

A very recent study reported that two functional SNPs (rs6122429 and rs2236196) in CHRNA4 were associated with luciferase activity (Winterer et al. 2007), and the question of whether these SNPs were associated in our schizophrenic samples should be examined. Of these SNPs, rs2236196 was included in this study, but rs6122429 was not. To evaluate whether our tag SNPs represent this rs6122429, located in the 5′ flanking region of CHRNA4, we genotyped rs6122429 using our 96 control samples. We found that this SNP was in LD with our SNP1 ($r^2 = 0.85$), and thus speculate that rs6122429 is not associated with schizophrenia in the Japanese population.

Our study is reasonable in terms of its design (selecting tag SNPs to represent each gene) and sample size large enough to gain high power. However, a couple of limitations should be noted. First, our samples were not age- or gender-matched. Although we included subgroup analyses divided by gender, careful interpretation is needed with respect to the association of schizophrenia itself. Second, we did not include a mutation scan to detect rare variants with functional effects. However, it is difficult to evaluate the association for a rare variant (e.g. MAF < 0.05). A larger sample size will be required for conclusive results in mutation search and association analysis. Lastly, several investigations have suggested that alternation of nAChRs may reflect the cognitive dysfunction and nicotine dependence associated with schizophrenia (Faraone et al. 2004; Levin and Simon 1998; Voineskos et al. 2007). Moreover, because the heritability of nicotine dependence and schizophrenia is reported to be about 40–70% (Li et al. 2003; Maes et al. 2004; Swan et al. 1990) and 80% (Cannon et al. 1998), respectively, we considered that the influence of genetic factors was about the same. Therefore, further study will be required to investigate the relationship between these genes and cognitive function or/and high smoking rate in schizophrenia, because we did not have information on smoking history in our samples or evaluate cognitive function.

In conclusion, our results suggest that *CHRNA4* and *CHRNB2* do not play a major role in schizophrenia in the Japanese population.

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