An association study of tachykinin receptor 3 gene with schizophrenia in the Japanese population

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The tachykinin receptor 3 (*TACR3*) gene encodes the neurokinin3 (NK3) receptor. Animal studies showed that agonist-induced stimulation of the NK3 receptor leads to the excessive release of dopamine in the ventral and dorsal striatal and prefrontal cortical regions. Data from clinical trials of selective NK3 receptor antagonists in schizophrenia have shown significant improvement in positive symptoms. We performed an association study of the *TACR3* gene in the Japanese population of 384 schizophrenic patients and 384 controls. Nine single nucleotide polymorphisms were genotyped using TaqMan assays and polymerase chain reaction-restriction fragment length polymorphism method. No significant association between schizophrenia and these single nucleotide polymorphisms was observed in single-marker and haplotype analyses. Our results suggest that *TACR3* is unlikely to be related to the development of schizophrenia in the Japanese population. *NeuroReport* 19:471–473 © 2008 Wolters Kluwer Health | Lippincott Williams & Wilkins.

Keywords: association study, dopamine, neurokinin3 receptor, schizophrenia, tachykinin receptor 3

Introduction

Schizophrenia is a severe, disabling and lifelong mental disorder with a global prevalence of 1%. Although it is generally accepted that genetic factors contribute to the development of this disease, its etiology has not yet been clarified.

The tachykinin receptor 3 (*TACR3*) gene located on chromosome 4q25 encodes the neurokinin 3 receptor (NK3). Its endogenous ligand is neurokinin B, a member of the tachykinin peptide family. It is expressed in the central nervous system and spinal cord [1]. Several animal studies have been performed investigating the interaction between NK3 receptors and dopamine (DA) and serotonin (5-HT) pathways. First, stimulation of NK3 receptors in the ventral mesencephalon increases DA release in the ventral and dorsal striatum and prefrontal cortex [2,3]. Second, infusion of NK3 receptor agonists into the ventral tegmental area evokes DA-mediated behaviors, such as yawning and chewing, which are potently inhibited by DA2 receptor antagonists such as haloperidol [4,5]. Third, injection of NK3 receptor agonists into the raphe area elicits 5-HT-mediated behaviors, such as head twitches, which are inhibited by 5-HT2A/5-HT2C receptor antagonists [6,7]. Additionally, a recent clinical study showed NK3 receptor antagonists improved the positive symptoms in schizophrenia [8]. These results suggest that altered form and function of the NK3 receptor might be related to the abnormalities of DA and 5-HT signaling, one of the major hypotheses explaining the pathophysiology of schizophrenia.

For all of these reasons, *TACR3* was hypothesized to be involved in the pathogenesis of schizophrenia. In this study, we performed linkage disequilibrium (LD) analysis of the *TACR3* gene and carried out case–control association studies between TACR3 polymorphisms and schizophrenia using single-marker association analysis and haplotype analysis in the Japanese population.

Methods

Participants

A total of 384 patients with schizophrenia [231 men, mean age ± standard deviation (SD) 48.8 ± 14.4; 153 women, 53.0 ± 15.9] and 384 controls (161 men, 40.8 ± 15.6; 223 women, 39.5 ± 13.8) were genotyped. All participants were ethnically Japanese and unrelated to each other. The schizophrenic patients, who were diagnosed according to the DSM-IV criteria with the consensus of at least two experienced psychiatrists on the basis of empirical diagnostic interviews and review of medical records, were recruited from several psychiatric hospitals around the Tokyo and Nagoya areas (within a 350 km radius). All healthy control participants with no current or past contact with psychiatric services were also screened on the basis of brief diagnostic interviews. This study was approved by the Ethics Committee of the Nagoya University Graduate School of Medicine, and written informed consent was obtained from each participant.

Haplotype tag single nucleotide polymorphism selection

We first consulted the HapMap database (release #16c.1, *www.hapmap.org*) and determined the LD block with the criteria $D' > 0.8$ using HAPLOVIEW ver. 3.2 software [9]. All single nucleotide polymorphisms (SNPs) listed in the entire coding region as well as the 500 bp upstream 5′-flanking region and 500 bp downstream 3′-UTR region (minor allele frequency > 0.05) were included in the LD analysis. Haplotype tag SNPs (htSNPs) were defined as those capturing 90% of the haplotype diversity within each LD block using the same program. The Japanese portion of the HapMap data was used for this procedure (Fig. 1).

Single nucleotide polymorphism genotyping

Genotyping of the htSNPs was carried out using TaqMan assays (Applied Biosystems, Foster City, California, USA) and the PCR-restriction fragment length polymorphism (RFLP) method (Table 1). TaqMan probes and Universal PCR Master Mix were obtained from Applied Biosystems. A 5-μl total reaction volume was used, and allelic-specific fluorescence was measured using the ABI PRISM 7900 Sequence Detector System (Applied Biosystems). Detailed information on the PCR method is available upon request.

Statistical analysis

Genotype deviation from the Hardy–Weinberg equilibrium (HWE) was evaluated by the χ^2 test. Single marker association and haplotype analyses were performed with SPSS version 11.0J (Tokyo, Japan) and COCAPHASE version 2.403 (*http://portal.litbio.org/Registered/Option/unphased*; Dudbridge, 2003), respectively. The significance level for all statistical tests was 0.05. Power calculations were performed using the genetic statistical package Genetic Power Calculator (*http://pngu.mgh.harvard.edu/~purcell/gpc/*; Purcell 2001–2005).

Results

The *TACR3* gene was composed of six LD blocks. One nonblock SNP and eight htSNPs were finally selected according to the criteria (Fig. 1). The genotype and allele frequency of each htSNP in schizophrenic patients and controls are summarized in Table 1. The observed genotype frequencies of all SNPs were within the distribution expected according to HWE. Neither the genotype nor allele frequency of any SNP differed significantly between the schizophrenia group and the control group (Table 1). The distribution of haplotype frequencies did not differ significantly between the schizophrenic patients and controls (Table 2). More than 80% power in detecting any association with schizophrenia was obtained when the genotype relative risk was set at 1.33–1.66 under a multiplicative model of inheritance.

Discussion

Our study indicates that the *TACR3* gene does not play a major role in the development of schizophrenia in the Japanese population, as no significant differences in allele, genotype, or haplotype frequencies of the selected SNPs were found between schizophrenic patients and controls. As it is, however, suspected that genetic risk factors for schizophrenia may differ between races or ethnicities, a replication study including different ethnic populations is needed to validate these results.

As mentioned in the Introduction, the NK3 receptor was reported to regulate the DA and 5-HT release or concentration at the synapse. It would therefore be valuable to investigate the gene–gene interactions between *TACR3* and other DA or 5-HT signaling related genes [10]. Furthermore, the 5-HT1A receptor partial agonist tandospirone is reported to be effective as an adjunctive treatment to improve cognition in patients with schizophrenia [11]. As the NK3 receptor is thought to have the potential for indirect influence on the 5-HT1A receptor through 5-HT release, association analysis using samples with data on the cognitive function might help elucidate the pathogenesis of schizophrenia.

A couple of limitations in this study should be considered. First, the male/female ratios and average ages did not match between schizophrenic patients and controls. When we performed a multiple regression analysis, there were no effects of age or sex on the disease status (data not shown). Additionally, these effects might be small because not likely

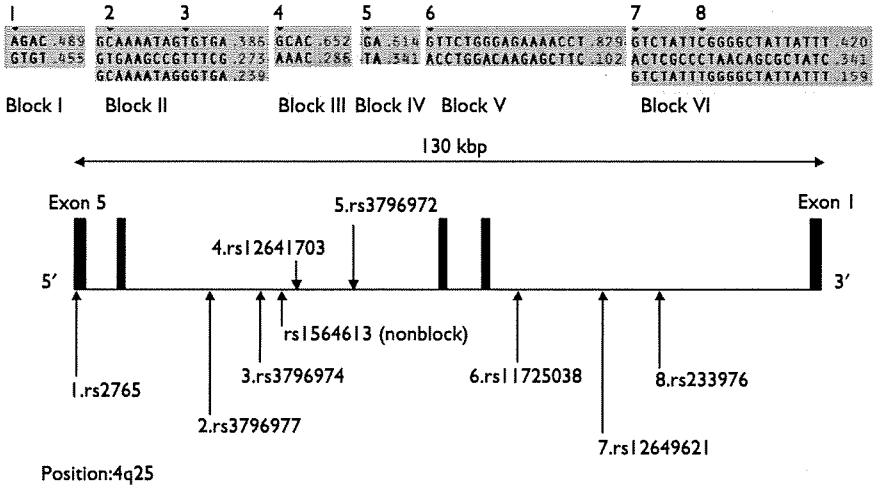

Fig. 1 Genomic structure of *TACR3* with haplotype tag single nucleotide polymorphisms (SNPs) and haplotype frequencies in each linkage disequilibrium block provided by HapMap database V.16. Numbers under or above the arrows represent the SNPs we selected in this study.

Table 1 Association analyses of haplotype tag SNPs

SNP	Block	Method of genotyping		GRR	Allelic distribution[a]			Genotypic distribution[a]			
					M	m	P value	M/M	M/m	m/m	P value
rs2765	I	TaqMan	SCZ	1.33	411	345	0.685	114	183	81	0.861
			CONT		401	351		112	178	87	
rs3796977	II	TaqMan	SCZ	1.41	611	153	0.471	249	113	20	0.79
			CONT		598	164		240	120	22	
rs3796974	II	TaqMan	SCZ	1.34	502	264	0.992	165	172	46	0.563
			CONT		493	259		169	156	52	
rs12641703	III	TaqMan	SCZ	1.35	506	260	0.669	167	172	44	0.536
			CONT		485	261		164	157	52	
rs3796972	IV	PCR-RFLP	SCZ	1.33	406	350	0.45	106	195	78	0.397
			CONT		414	330		121	177	77	
rs11725038	V	TaqMan	SCZ	1.37	556	210	0.899	204	149	31	0.954
			CONT		548	210		201	146	33	
rs12649621	VI	TaqMan	SCZ	1.33	430	336	0.284	127	178	79	0.53
			CONT		407	355		113	183	87	
rs233976	VI	TaqMan	SCZ	1.44	639	123	0.833	270	100	12	0.614
			CONT		642	120		269	106	8	
rs1564613	Non-block	TaqMan	SCZ	1.66	710	52	0.799	331	48	2	0.793
			CONT		705	49		332	43	3	

CONT, control; GRR, genotype relative risk; M, major allele; m, minor allele; SCZ, schizophrenia; SNP, single nucleotide polymorphism.
[a]In absolute numbers.

Table 2 Haplotype analyses

Block	SNP	Haplo-type	SCZ[a]	CON[a]	P value[b]	Global P value[b]
		AT	0.455	0.44	0.558	
2	rs3796977-rs3796974	AC	0.345	0.344	0.993	0.734
		CT	0.2	0.215	0.467	
		GG	0.439	0.466	0.284	
6	rs12649621-rs233976	AG	0.4	0.377	0.348	0.531
		AA	0.161	0.157	0.837	

CONT, control; SCZ, schizophrenia; SNP, single nucleotide polymorphism.
[a]Estimated frequencies.
[b]P values were calculated by log-likelihood ratio test.

more than four participants given a lifetime morbidity risk of 1% will eventually develop schizophrenia. Second, we selected htSNPs so as to cover 90% of the haplotypes within each LD block. It is, however, possible that the htSNPs used in this study did not capture all haplotypes in the gene, as the LD block structure of *TACR3* was not tight. In other words, there may be SNPs not found in the LD, for which we did not investigate the possible association with schizophrenia. Thus, further analysis based on more comprehensive and detailed SNP coverage of *TACR3* is required to make conclusive results.

Conclusion

The present results suggest that *TACR3* itself is unlikely to be related to the development of schizophrenia in the Japanese population. Further studies including pharmacogenetic investigations are required, however, for conclusive results on the exact roles of *TACR3* in the pathophysiology of schizophrenia.

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Letter to the Editor

Large-scale case-control study of a functional polymorphism in the *glutamate receptor, metabotropic 3* gene in patients with schizophrenia

THE A ALLELE of rs6465084, a single nucleotide polymorphism (SNP) of the *glutamate receptor, metabotropic 3* (*GRM3*) gene, has been found to be associated with decreased verbal fluency and reduced prefrontal cortical levels of N-acetylaspartate/creatine.[1] This functional SNP has been shown to be associated with schizophrenia[1] but other studies failed to demonstrate such an association.[2–5] To further investigate the inconsistent results, we conducted a case–control association study.

The Ethics Committee on Genetics of each participating institute approved the present study. All participants provided written informed consent. All participants were unrelated Japanese subjects. The subjects consisted of 2358 patients with schizophrenia, meeting the DSM-IV criteria (1273 men, 1085 women; mean age 46.8 ± 14.7 years), and 2433 control subjects (1313 men, 1120 women; mean age 45.6 ± 13.8 years). We genotyped rs6465084 (C_11245618_10; Applied Biosystems, CA, USA) using the TaqMan 5′-exonuclease assay.[6] We did not examine rs1468412,[7] rs2299225[8] and rs274622[9] because these SNP have not been shown to be associated with schizophrenia in a large Japanese sample,[5] which overlapped the subjects in the present study.

The genotype distributions did not deviate significantly from Hardy–Weinberg equilibrium in either group. The genotype and allele frequencies in patients did not differ from those in controls (Table 1). The results of the present study indicate that the rs6465084 functional polymorphism in *GRM3* does not contribute to genetic susceptibility to schizophrenia.

Table 1 Genotype and allele frequencies of rs6465084 in *GRM3*

	Patients (%)	Controls (%)	*P*
Genotype			0.39
A/A	2045 (86.7)	2132 (87.6)	
A/G	305 (12.9)	289 (11.9)	
G/G	8 (0.4)	12 (0.5)	
Allele			0.46
A	4395 (93.2)	4553 (93.6)	
G	321 (6.8)	313 (6.4)	

GRM3, glutamate receptor, metabotropic 3.

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Genetic analysis of the gene coding for DARPP-32 (*PPP1R1B*) in Japanese patients with schizophrenia or bipolar disorder

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Abstract

Several lines of evidence, including genome-wide linkage scans and postmortem brain studies of patients with schizophrenia or bipolar disorder, have suggested that DARPP-32 (dopamine- and cAMP-regulated phosphoprotein, 32 kDa), a key regulatory molecule in the dopaminergic signaling pathway, is involved in these disorders. After evaluating the linkage disequilibrium pattern of the gene encoding DARPP-32 (*PPP1R1B*; located on 17q12), we conducted association analyses of this gene with schizophrenia and bipolar disorder. Single-marker and *haplotypic* analyses of four single nucleotide polymorphisms (SNPs; rs879606, rs12601930, rs907094, and rs3764352) in a sample set (subjects with schizophrenia=384, subjects with bipolar disorder=318, control subjects=384) showed that *PPP1R1B* polymorphisms were not significantly associated with schizophrenia, whereas, even after Bonferroni corrections, significant associations with bipolar disorder were observed for rs12601930 (corrected genotypic $p=0.00059$) and rs907094 (corrected allelic $p=0.040$). We, however, could not confirm these results in a second independent sample set (subjects with bipolar disorder=366, control subjects=370). We now believe that the significant association observed with the first sample set was a result of copy number aberrations in the region surrounding these SNPs. Our findings suggest that *PPP1R1B* SNPs are unlikely to be related to the development of schizophrenia and bipolar disorder in the Japanese population.

Keywords: Schizophrenia; Bipolar disorder; Dopamine- and cAMP-regulated phosphoprotein; 32 kDa; Japanese population

1. Introduction

A number of studies have proposed that disruption of monoaminergic pathways, and in particular the dopaminergic pathway, contributes to both schizophrenia and bipolar disorder (Catapano and Manji, 2007; Murray

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et al., 2004). DARPP-32 (dopamine- and cAMP-regulated phosphoprotein, 32 kDa), a critical molecule in *the* striatal neurons, regulates the dopaminergic signaling pathway through phosphorylation of protein phosphatase-1 and protein kinase A (Fienberg et al., 1998). Recently, it has been revealed that DARPP-32 also plays an important role in the regulation of *glutamatergic* signaling pathway (Nishi et al., 2005), which is also thought to contribute to the development of these disorders (Beneyto et al., 2007; Svenningsson et al., 2003).

DARPP-32 knockout mice have been shown to have abnormal responses to psychoactive drugs, such as the decrease of cage climbing behavior induced by dopamine agonists (Fienberg et al., 1998) and the decrease of attenuating effect of antidepressants on immobility (Svenningsson et al., 2002).

Moreover, reduced expression of DARPP-32 has been observed in the postmortem brain of schizophrenic patients (Albert et al., 2002). This is suggested to be related to neostriatal volume, activation, and functional connectivity in the prefrontal cortex, all of which are thought to be abnormal in patients with schizophrenia (Meyer-Lindenberg et al., 2007).

Additionally, several lines of evidence have demonstrated that genetic factors contribute to the development of schizophrenia and bipolar disorder, and genome-wide linkage scans have shown that several chromosomal regions are simultaneously linked to the development of these disorders. Namely, a chromosomal region within 17q, which includes the gene encoding DARPP-32 (*PPP1R1B*; located on 17q12), has been demonstrated to have high logarithm of the odds scores for schizophrenia (Cardno et al., 2001) and bipolar disorder (Dick et al., 2003), i.e. 2.54 and 3.63, respectively.

Therefore, *PPP1R1B* is considered to be one of the candidate genes that contribute to these disorders. In the present study, we performed linkage disequilibrium analysis of *PPP1R1B*, and investigated the association of polymorphisms in this gene with schizophrenia and bipolar disorder in Japanese patients. We employed a two-stage analysis using two independent sets of samples as a previous report (Ikeda et al., 2005). Additionally, copy number variations (CNVs), which have been observed for many genes (Lee and Lupski, 2006; Redon et al., 2006) can affect the accuracy of genotyping with single nucleotide polymorphisms (SNPs). Therefore, we also explored copy number differences of this gene to test the accuracy of genotyping with the SNPs, which deviated from the Hardy–Weinberg equilibrium (HWE).

2. Materials and methods

2.1. Subjects

The subjects for the case-control analysis consisted of 384 patients with schizophrenia (226 males and 158 females; 52.1±15.3 years old), 318 patients with bipolar disorder (162 males and 156 females; 44.0±20.7 years old), and 384 control subjects (159 males and 225 females; 43.9±15.9 years old). To confirm a significant association with bipolar disorder, a second sample set was used, which consisted of 366 patients with bipolar disorder (181 males and 185 females; 50.1±13.4 years old), and 370 control subjects (185 males and 185 females; 50.6±12.6 years old).

For the analysis of copy number differences, we selected 12 male and 12 female subjects (schizophrenia patients: 54.9±12.5 years old; bipolar disorder patients: 46.3±17.4 years old; control subjects: 43.0±12.6 years old) for screening, and another independent sample set of 36 male and 36 female subjects (schizophrenia patients: 41.1±12.2 years old; bipolar disorder patients: 47.1±15.6 years old; control subjects: 43.5±12.7 years old) was used to confirm the results. The subjects used for the copy number analysis were also included in the first sample set used for the association study.

All subjects were unrelated and ethnically Japanese. The patients were diagnosed by at least two experienced psychiatrists according to the DSM-IV-TR (Diagnostic and Statistical Manual of Mental Disorders, Fourth Edition, Text Revision) criteria for schizophrenia and bipolar disorder on the basis of unstructured interviews and reviews of their medical records. All healthy control subjects were also psychiatrically screened on the basis of unstructured interviews.

This study was approved by the Ethics Committees of the Nagoya University Graduate School of Medicine, Fujita Health University, and the RIKEN Brain Science Institute. Written informed consent was obtained from each subject.

2.2. Linkage disequilibrium (LD) analysis and tagging SNP selection

For LD analysis, we consulted the HapMap database (release #21a; population: Japanese in Tokyo; minor allele frequency: more than 0.05) in order to obtain SNPs throughout the entire coding region of *PPP1R1B* (GenBank accession No. NM_032192) as well as in flanking regions 500 base pairs (bp) upstream and 500 bp downstream of the coding regions. For the gene-spanning analysis, we selected four SNPs (rs2271309,

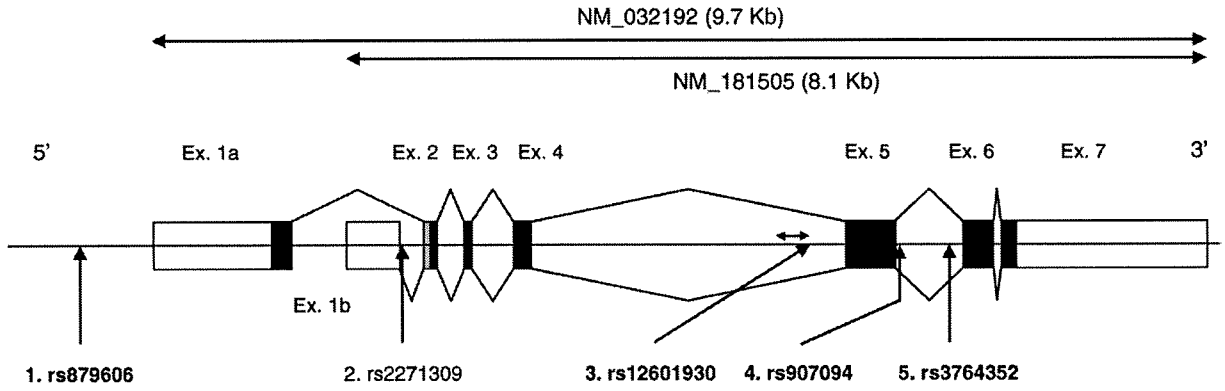

Fig. 1. Genomic structure of *PPP1R1B*. Black boxes indicate protein-coding regions, whereas open boxes denote untranslated regions (UTR). Each box represents the *PPP1R1B* exons. Numbers under the arrows represent the SNP IDs. Bold numbers represent tagging SNPs (pairwise tagger; $r^2>0.8$; Haploview 3.32). All SNPs in the coding region are listed as well as those within the 500-bp upstream 5′-flanking region and the 500-bp downstream 3′ UTR of *PPP1R1B*. Arrows ~200 bp upstream of rs12601930 shows the sites that were PCR amplified for copy number analysis.

rs12601930, rs907094, and rs3764352) in addition to a dbSNP (rs879606) located in the promoter region (Fig. 1). After evaluating the LD pattern with 48 control subjects using Haploview version 3.32, rs2271309 was excluded according to the criterion for pairwise tagging, $r^2>0.8$ (de Bakker et al., 2005) (see Fig. 2).

2.3. SNP genotyping

Genotyping of tagging SNPs was carried out primarily using polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) analysis. TaqMan assays were used when available (Applied Biosystems, California, USA). TaqMan probes and Universal PCR Master Mix were obtained from Applied Biosystems. Allelic-specific fluorescence was measured using an ABI PRISM 7900 Sequence Detector System (Applied Biosystems). Information about each primer pair and enzyme is available upon request.

2.4. Quantitative real-time PCR

The *PPP1R1B* copy number was analyzed using real-time PCRs with a specific primer set and FAM-labeled fluorescent probe or TaqMan expression assays (Applied Biosystems). The test region for *PPP1R1B* was ~200 bp upstream of the SNP that produced results that deviated from the HWE (Fig. 1) and the prostaglandin transporter gene (*SLCO2A1*) was used as a single-copy control gene (Wilson et al., 2006). We did not use glucose-6-phosphate dehydrogenase (G6PD), which is located on the X chromosome and was used as single-copy control gene in the study from Wilson et al., because it showed an unstable copy number value (not an integral value) in their report.

To determine the relative copy number, 10 ng of genomic DNA was assayed in triplicate in 20 μL of reaction solution containing 1 × final concentration TaqMan Universal Master Mix (Applied Biosystems) and 1 × final concentration TaqMan probe (Applied Biosystems) specific for *PPP1R1B* or 700 nM each primer and 200 nM probe specific for *SLCO2A1*.

Each experiment was performed using an ABI PRISM 7900 Sequence Detector System (Applied Biosystems). To evaluate the relative copy number of *PPP1R1B*, we calculated the relative quantity of the dose of *PPP1R1B* using a comparative C_T method (*PPP1R1B* vs. *SLCO2A1*). The TaqMan specific primers and FAM-labeled fluorescent probe used for the PCR amplifications were as follows: *PPP1R1B*-FAM probe (5′-FAM-CCCCTTGCTCCTTTCC-MGBNFQ-3′), *PPP1R1B*-for (5′-GCCTTGGCCCCTTTCTCTAA-3′), *PPP1R1B*-rev (5′-GCAGCTGGAGACAAGTTTCC-3′), *SLCO2A1*-FAM (5′-FAM-CCATCCATGTCCTCATCTC-

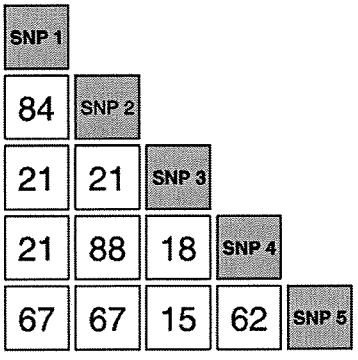

Fig. 2. LD analysis of *PPP1R1B*. Numbers in the top gray boxes correspond to the SNP ID numbers in Fig. 1. Numbers in the white boxes represent the r^2 values after the decimal point.

Table 1
Genotype and allele frequency of *PPP1R1B* in schizophrenia and controls

Gene symbol	SNP ID (M/m)	Method of genotyping	Genotype[a]					*p* value	Armitage's	Allele			*p* value	Global
			SCZ[b]			CON[b]			*p* value	SCZ	CON			*p* value[c]
PPP1R1B	rs879606 (G/A)	RFLP	G/G 62 (34%)	G/A 81 (45%)	A/A 39 (21%)	G/G 58 (32%)	G/A 91 (49%)	A/A 35 (19%)	0.631	0.985	G 205 (56%)	A 159 (44%)	G 207 (56%)	A 161 (44%)	0.985	0.697
	Rs12601930 (C/T)	real time PCR	C/C 256 (68%)	C/T 110 (29%)	T/T 11 (3%)	C/C 240 (63%)	C/T 134 (35%)	T/T 8 (2%)	0.190	0.271	C 622 (83%)	T 132 (18%)	C 614 (80%)	T 150 (20%)	0.287	
	rs907094 (T/C)	real time PCR	T/T 99 (27%)	T/C 202 (54%)	C/C 71 (19%)	T/T 114 (30%)	T/C 193 (50%)	C/C 77 (20%)	0.518	0.673	T 400 (54%)	C 344 (46%)	T 421 (55%)	C 347 (45%)	0.681	
	rs3764352 (A/G)	RFLP	A/A 86 (24%)	A/G 193 (54%)	G/G 77 (22%)	A/A 104 (28%)	A/G 195 (52%)	G/G 79 (21%)	0.582	0.419	A 365 (51%)	G 347 (49%)	A 403 (53%)	G 353 (47%)	0.434	

[a]M: major allele, m: minor allele [b]SCZ: schizophrenia, CON: control [c]global *p* value: haplotypic analysis.

MGBNFQ-3′), *SLCO2A1*-for (5′-ATCCCCAAAG-CACCTGGTTT-3′), and *SLCO2A1*-rev (5′-AGAGGC-CAAGATAGTCCTGGTAA-3′).

2.5. Statistical analysis

Genotype deviations from the HWE and single-marker association were analyzed using Haploview software. We evaluated the allelic and genotypic associations by the χ^2-test. Genotypic association of SNPs that deviated from the HWE was analyzed using Cochran-Armitage trend tests for multiplicative model of inheritance (Balding, 2006). Haplotypic analyses were performed with Unphased version 2.403 (Dudbridge, 2003). The significance level for all statistical tests was 0.05. Bonferroni corrections were used for multiple comparisons. Power calculations were performed using the genetic statistical package on a genetic power calculator (Purcell et al., 2003).

3. Results

The genotype and allele frequency of each SNP in schizophrenic patients, bipolar disorder patients, and control subjects are summarized in Table 1 and Table 2-1, respectively. The observed genotype frequencies of the tagging SNPs were within the distribution expected from the HWE except for rs12601930.

Neither the genotype nor the allele frequency of any of the examined *PPP1R1B* SNPs in the schizophrenic patients differed significantly from those observed for the control subjects (Table 1). Moreover, the distribution of haplotypic frequencies in the schizophrenia patients did not differ significantly from that in control subjects. Power analysis showed that more than 80% power in detecting an association with schizophrenia was obtained when the genotype relative risk (GRR) was set from 1.35 to 1.51 in a multiplicative model of inheritance.

A significant association was observed with both the genotype and the allele containing rs907094 ($p=0.036$ and $p=0.010$, respectively), whereas a significant association was only found with the genotype for rs12601930 ($p=0.000147$). Haplotypic analysis supported this association (global $p=0.030$; Table 2-1). After Bonferroni corrections, the observed positive associations were no longer significant for the rs907094 genotype (corrected $p=0.144$) and haplotype (corrected global $p=0.120$), whereas, even after the corrections, the associations remained significant for the rs907094 allele (corrected $p=0.040$) and the rs12601930 genotype (corrected $p=0.000588$). We, however, could not confirm these

Table 2-1
Genotype and allele frequency of *PPP1R1B* in bipolar disorder and controls (first sample set)

Gene symbol	SNP ID (M/m)	Method of genotyping	Genotype[a]						*p* value	Armitage's	Allele				*p* value	Global
			BP[b]			CON[b]				*p* value	BP		CON			*p* value[c]
PPP1R1B	rs879606 (G/A)	RFLP	G/G 87 (30%)	G/A 146 (50%)	A/A 61 (21%)	G/G 58 (32%)	G/A 91 (49%)	A/A 35 (19%)	0.857	0.580	G 320 (54%)	A 268 (46%)	G 207 (56%)	A 161 (44%)	0.306	0.030
	rs12601930 (C/T)	real time PCR	C/C 169 (69%)	C/T 57 (23%)	T/T 18 (7%)	C/C 240 (63%)	C/T 134 (35%)	T/T 8 (2%)	0.000147	0.804	C 395 (81%)	T 3 (19%)	C 614 (80%)	T 150 (20%)	0.802	
	rs907094 (T/C)	real time PCR	T/T 63 (23%)	T/C 202 (54%)	C/C 71 (19%)	T/T 114 (30%)	T/C 193 (50%)	C/C 77 (20%)	0.036	0.010	T 267 (48%)	C 293 (52%)	T 421 (55%)	C 347 (45%)	0.010	
	rs3764352 (A/G)	RFLP	A/A 64 (22%)	A/G 170 (58%)	G/G 58 (20%)	A/A 104 (28%)	A/G 195 (52%)	G/G 79 (21%)	0.176	0.385	A 298 (51%)	G 286 (49%)	A 403 (53%)	G 353 (47%)	0.407	

[a]M: major allele, m: minor allele [b]BP: bipolar disorder, CON: control [c]global *p* value: haplotypic analysis.

Table 2-2
Genotype and allele frequency of *PPP1R1B* in bipolar disorder and controls (second sample set)

Gene symbol	SNP ID (M/m)	Method of genotyping	Genotype[a]						*p* value	Armitage's	Allele				*p* value	Global
			BP[b]			CON[b]				*p* value	BP		CON			*p* value[c]
PPP1R1B	Rs12601930 (C/T)	real time PCR	C/C 232 (63%)	C/T 119 (33%)	T/T 15 (4%)	C/C 248 (68%)	C/T 105 (29%)	T/T 12 (3%)	0.419	0.192	C 583 (80%)	T 149 (20%)	C 601 (82%)	T 129 (18%)	0.191	0.307
	Rs907094 (T/C)	real time PCR	T/T 102 (28%)	T/C 190 (53%)	C/C 70 (19%)	T/T 107 (29%)	T/C 180 (49%)	C/C 81 (22%)	0.565	0.731	T 394 (54%)	C 330 (46%)	T 394 (54%)	C 342 (46%)	0.734	

[a]M: major allele, m: minor allele [b]BP: bipolar disorder, CON: control [c]global *p* value: haplotypic analysis.

Table 3-1
PPP1R1B copy number variations detected by qPCR[a] (first sample set)

	SCZ[b] (n=24)	BP[b] (n=24)	CON[b] (n=24)
Relative quantity	0.81 ± 0.14	0.87 ± 0.10	1.11 ± 0.34
Decrease	6	3	2
Normal	17	21	15
Increase	1	0	7

[a]qPCR: quantitative real-time PCR.
[b]SCZ: schizophrenia, BP: bipolar disorder, CON: control.

results in a second independent sample set as shown in Table 2-2. When the two sample sets were merged, power analysis showed the level for detecting association was higher than 80% for bipolar disorder at the genotype relative risk of 1.24 to 1.39 under a multiplicative model of inheritance.

DNA copy number analysis revealed aberrations in the copy number in the schizophrenic patients (increase=1 patient, decrease=6), the bipolar patients (decrease=3), and the control subjects (increase=7, decrease=2; Table 3-1). In the second independent sample set, we observed aberrations in the schizophrenic patients (increase=2, decrease=1), the bipolar patients (increase=2, decrease=1), and the control subjects (increase=2, decrease=5; Table 3-2). All analyses were performed in duplicate.

4. Discussion

According to the common disease-common variants hypothesis (Chakravarti, 1999), the present study showed that *PPP1R1B* was unlikely to be related to be the development of schizophrenia and bipolar disorder in Japanese patients. These results were consistent with a recently published study that examined Chinese patients (Li et al., 2006).

The SNPs used in the association analysis, which covered the entire gene, included all of the common SNPs (more than 5% frequency) listed in the dbSNP database; therefore, it is unlikely that there are other common variants related to these disorders. Because we did not perform mutation screening of this gene, however, the possibility that rare variants could be causal to the development of these disorders cannot be excluded. The GRR value calculated using power analysis was appropriate when compared to other promising candidate genes for schizophrenia (Schwab et al., 2005; Schwab et al., 2003; Shifman et al., 2002).

Recently, Meyer-Lindenberg et al. (2007) tested for an association in schizophrenia with SNPs in this gene using a relatively small sample of Caucasian families, and found a strong association with rs879606 and mild association with two other SNPs (rs3764352 and rs3794712). Two of these three SNPs (rs879606 and rs3764352), however, did not show any significant association in our samples, suggesting that ethnic differences might play a role in these associations.

rs3794712 was not further pursued for two reasons. *Firstly*, this SNP is not validated and the frequency of this SNP is not reported in the dbSNP database. *Secondly*, this SNP is unlikely to affect the function of this gene considering its genomic position in the middle of an intron and the results from *in silico* analysis (RegRNA; http://regrna.mbc.nctu.edu.tw/index.html).

In the present study, we found that copy number differences in the region that includes the SNPs deviated from the HWE and were significantly associated with bipolar disorder (Tables 3-1 and 3-2), suggesting that the presence of copy number alterations gives rise to the deviation from the HWE due to a high frequency of heterozygotes. Therefore, caution must be taken when interpreting results from SNP analysis. These results, however, must be regarded as preliminary because we did not observe a definite association between copy number differences and the disorders. Because accumulating evidence has highlighted that CNVs are observed in many chromosome regions, *including 17q12* (Sharp et al., 2006), and copy number alterations can affect gene expression (Redon et al., 2006), further investigations of the CNVs as well as the SNPs may be an effective complementary approach to elucidate the genetic risk factors underlying the complex phenotypes associated with psychiatric disorders, including schizophrenia and bipolar disorder (Lee and Lupski, 2006).

A couple of limitations should be considered in the present study. Firstly, we observed a strong association between rs12601930 and bipolar disorder in the first sample set even after a Bonferroni correction (corrected genotype p=0.000588); the genotype frequency of the minor allele homozygotes (TT) in the control subjects, however, was only 2% (Table 2-1). This low frequency may have resulted in a false-positive association. When we used a Cochran–Armitage trend test (Balding, 2006)

Table 3-2
PPP1R1B copy number variations detected by qPCR[a] (second sample set)

	SCZ[b] (n=24)	BP[b] (n=24)	CON[b] (n=24)
Relative quantity	0.95 ± 0.10	0.98 ± 0.14	1.03 ± 0.27
Decrease	2	2	2
Normal	69	69	65
Increase	1	1	5

[a]qPCR: quantitative real-time PCR.
[b]SCZ: schizophrenia, BP: bipolar disorder, CON: control.

to correct the observed association with rs12601930, which deviated from the HWE, there was no significance in the genotype frequency (Armitage's $p=0.804$). Thus, the possibility of a type I error derived from an insufficient sample size should be considered. Secondly, although the method used for copy number analysis has been widely used and is thought to be highly reliable due to the consistency of results obtained in our duplicate experiments, supplementary methods, such as Southern blotting, fluorescence in situ hybridization, or array comparative genomic hybridization, would have provided further confirmation of our results. Finally, the male/female ratios and average ages were not completely consistent between the schizophrenic patients and the control subjects. Based on results from an exploratory analysis using a logistic regression model, however, these variables do not appear to contribute to the results obtained in the present association study (data not shown).

In conclusion, our findings suggest that SNPs within *PPP1R1B* do not elevate the risk for either schizophrenia or bipolar disorder in the Japanese population. Further functional analysis of the CNVs and association studies using other endophenotypes including cognitive function should be needed to clarify the exact role of this gene in the pathophysiology of these disorders.

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Contributors

Author Akira Yoshimi, Nagahide Takahashi, and Shinichi Saito designed the study and wrote the protocol. Author Norio Ozaki and Yukihiro Noda performed the literature searches and analyses. Author Akira Yoshimi wrote the first draft of the manuscript and Nagahide Takahashi and Shinichi Saito revised it. All of the authors contributed to and have approved the final version of the manuscript.

Conflict of interest

All authors declare that they have no conflicts of interests.

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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.

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

		The A allele of rs1468412					The G allele of rs2299225				
References	Population	Patients with schizophrenia (n)	Control (n)	OR	(95% CI)	*P*	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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ORIGINAL ARTICLE

Microarray comparative genomic hybridization analysis of 59 patients with schizophrenia

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Abstract Schizophrenia is a common psychiatric disorder with a strong genetic contribution. Disease-associated chromosomal abnormalities in this condition may provide important clues, such as *DISC1*. In this study, 59 schizophrenia patients were analyzed by microarray comparative genomic hybridization (CGH) using custom bacterial artificial chromosome (BAC) microarray (4,219 BACs with 0.7-Mb resolution). Chromosomal abnormalities were found in six patients (10%): 46,XY,der(13)t(12;13)(p12.1; p11).ish del(5)(p11p12); 46,XY, ish del(17)(p12p12); 46,XX.ish dup(11)(p13p13); and 46,X,idic(Y)(q11.2); and in two cases, mos 45,X/46XX. Autosomal abnormalities in three cases are likely to be pathogenic, and sex chromosome abnormalities in three follow previous findings. It is noteworthy that 10% of patients with schizophrenia have (sub)microscopic chromosomal abnormalities, indicating that genome-wide copy number survey should be considered in genetic studies of schizophrenia.

Keywords Schizophrenia · Chromosomal abnormality · Array comparative genomic hybridization · Copy number variation

Introduction

Schizophrenia is a common psychiatric disorder involving approximately 1% of the population worldwide. Family, twin, and adoption studies suggest genetic factors contribute to this illness (Lang et al. 2007; McGuffin et al. 1995). Meta-analysis including 18 genome scans revealed strong evidence at chromosomal regions 22q, 8p, and 13q

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as the susceptibility loci (Badner and Gershon 2002), and another meta-analysis of 20 genome-wide scans suggested regions of chromosomes 2q, 5q, 3p, 11q, 6p, 1q, 22q, 8p, 20q, and 14p as the significant loci (Lewis et al. 2003). Chromosomal abnormalities in patients with schizophrenia may provide useful information regarding the susceptible loci (Bassett et al. 2000). Disrupted in schizophrenia 1 (*DISC1*) gene isolated from a large Scottish family with t(1;11)(q42.1;q14.3) and high risk of schizophrenia in velo-cardio-facial syndrome (VCFS) with a 22q11 deletion are good examples (Arinami 2006; Millar et al. 2000; Murphy 2002). Some linkage and association studies support that schizophrenia could be associated with *DISC1* and genes at 22q11 (Chubb et al. 2008; Liu et al. 2002; O'Donovan et al. 2003; Shifman et al. 2002).

Microarray technologies have now become practical tools for detection of submicroscopic copy number changes. Using custom bacterial artificial chromosome (BAC) microarray (4,219 BACs at 0.7-Mb resolution), we analyzed 59 patients with schizophrenia. Chromosomal abnormalities found in this study are presented.

Materials and methods

Subjects

A total of 59 subjects (31 men and 28 women) with schizophrenia were recruited in this study. Forty-one had family history. Diagnosis was made for each patient according to the *Diagnostic and Statistical Manual of Mental Disorders*, 4th edition (DSM-IV) criteria on the basis of unstructured interviews and information from medical records. Participants were excluded if they had organic brain diseases, including head injury and infection, or if they met criteria for alcohol/drug dependence. After written informed consent, genomic deoxyribonucleic acid (DNA) from lymphoblastoid cell line (LCL) of all patients was isolated using DNA isolation systems [Quick Gene-800 (Fujifilm, Tokyo, Japan) and/or NA-3000 (Kurabo, Osaka, Japan)]. Micorarray comparative genomic hybridization (CGH) and fluorescence in situ hybridization (FISH) analysis were performed using materials from LCL. Peripheral blood lymphocytes were reevaluated in ID394, MZ102, and MZ127, but could not be obtained for reexamination in ID67, ID345, or ID391. Only parents of ID345 subjects were available for familial analysis. Other parents or sibs could not be evaluated. Experimental protocols were approved by the Committee for Ethical Issues at Yokohama City University School of Medicine.

Microarray CGH analysis

Comparative genomic hybridization analysis was performed using our custom BAC microarray containing 4,219 BAC clones, as previously described (Saitsu et al. 2008). In brief, after complete digestion using *Dpn*II, subject's DNA was labeled with Cy-5 dCTP (Amersham Biosciences, Piscataway, NJ), and reference DNA was labeled with Cy-3 deoxycytidine triphosphate (dCTP) (Amersham Biosciences) using the DNA random primer Kit (Invitrogen). Prehybridization, probe hybridization, washing, and drying steps for arrays were preformed on a Tecan hybridization station HS400 (Tecan Japan, Kawasaki, Japan). Arrays were scanned by GenePix 4000B (Axon Instruments, Union City, CA, USA) and analyzed using GenePix Pro 6.0 (Axon Instruments). The signal intensity ratio between patient and control DNA was calculated from the data of the single-slide experiment using the ratio of means formula (F635 mean − B635 median/F532 mean − B532 median) according to GenePix Pro. 6.0. The standard deviation was calculated from the data of all clones. We regarded the signal ratio as abnormal if it ranged out of ±3 standard deviations (SD). Clones showing abnormal copy number were checked to see whether they were in the position of previously registered copy number variations using the Human Genome Variation Database (http://www.hgvbase.org/) (Iafrate et al. 2004). Unregistered changes were considered for further confirmation. Genome position was based on the UCSC genome browser Human Mar. 2006 (hg18) assembly.

Fluorescence in situ hybridization

To confirm status of clones with a possibly abnormal copy number, FISH was performed, as previously described (Shimokawa et al. 2005). BAC DNA was labeled with SpectrumGreen™-11-deoxyuridine triphosphate (dUTP) or SpectrumOrange™-11-dUTP (Vysis, Downers Grove, IL, USA) by nick translation and denatured at 70°C for 10 min. Probe-hybridization mixtures (15 μl) were applied on chromosomes, incubated at 37°C for 16–72 h, then washed and mounted in antifade solution (Vector, Burlingame, CA, USA) containing 4'-6'-diamidino-2-phenylindole (DAPI). Photographs were taken on an AxioCam MR CCD fitted to Axioplan2 fluorescence microscope (Carl Zeiss, Oberkochen, Germany). In ID394 and MZ102, we counted 100 interphase nuclei to validate the number of cells with X aneuploidy, as well as 30 metaphases.

Results and discussion

Six patients showed chromosomal abnormalities (10%, 6/59) (Table 1). As we could not obtain materials from most