Table 2
Logistic regression analysis of single markers in *HTR1A* with schizophrenia and bipolar disorder.

SNP[a]	Genotype	Schizophrenia			Bipolar disorder		
		OR[b]	95% CI[c]	*P*-value	OR[b]	95% CI[c]	*P*-value
rs6295	CC (reference)						
C>G	CG	2.09	0.932–4.59	0.0682	1.03	0.706–1.50	0.886
	GG	1.34	0.534–3.18	0.518	1.08	0.742–1.57	0.702
rs878567	CC (reference)						
C>T	CT	1.98	0.105–1.20	0.0667	1.00	0.648–1.54	0.993
	TT	2.91	0.589–16.3	0.201	1.90	0.930–3.89	0.0782

Reference genotypes are common genotype. Adjustment for age and gender.
[a] Major allele>minor allele.
[b] OR: odds ratio.
[c] CI: confidence interval.

wise analysis with the chi-square test (Table 1), but not with logistic regression adjusted for age and gender (Table 2). In addition, we found an association between *HTR1A* and BP in the haplotype-wise analysis adjusted for age and gender (Table 3). In the power analysis, we obtained power of more than 80% for the detection of association when we set the genotype relative risk at 1.20–1.26 in BP for *HTR1A*, under a multiplicative model of inheritance.

3.2. Meta-analysis

3.2.1. Schizophrenia

In the meta-analysis, two association studies, including our study, met our criteria for rs6295 (Table 4). We found significant heterogeneity among ORs ($P(Q) = 0.000142$). The pooled OR derived from all studies comprising 965 patients and 1964 healthy control subjects did not indicate a significant association (random model: pooled OR = 0.793, 95% CI = 0.387–1.623, $P(Z) = 0.526$) (Fig. 1).

3.2.2. Bipolar disorder

In the meta-analysis, three association studies, including our study, met our criteria for rs6295 (Table 4).We did not find significant heterogeneity among ORs ($P(Q) = 0.789$). The pooled OR derived from all studies comprising 1148 patients and 1964 healthy control subjects indicated a significant association (fixed model: pooled OR = 0.794, 95% CI = 0.641–0.983, $P(Z) = 0.0344$) (Fig. 1). No publication bias was found ($t = 0.656$, $p = 0.536$).

4. Discussion

Although we detected an association between rs878567 and schizophrenia in the allele-wise analysis, this significance disappeared after multiple testing. We did not detect a significant association between *HTR1A* and schizophrenia in the genotype-wise analysis or haplotype-wise analysis with logistic regression adjusted for age and gender (Tables 2 and 3). Therefore, our results suggest that *HTR1A* does not play a role in the pathophysiology of schizophrenia in the Japanese population. On the other hand, in the single-marker association study, we detected a significant association between *HTR1A* and BP with the chi-square test. However, this association may have been due to biased samples, which were unmatched for gender. We therefore performed a logistic regression analysis to compare the phenotypes of each of the examined SNP genotypes, using several clinical factors as other independent variables to adjust for possible confounding. Although we did not detect an association between the two SNP genotypes in *HTR1A* and BP with logistic regression analysis, we found an association between *HTR1A* and BP in the haplotype-wise analysis adjusted for age and gender. Our results, therefore, suggest that *HTR1A* plays a role in the pathophysiology of BP in the Japanese population.

We detected an association between *HTR1A* and BP, but not schizophrenia. Ivleva and colleagues suggested that genes, which are associated with schizophrenia but not BP, may play a major role in the pathophysiology of psychosis. Genes associated with BP, but not schizophrenia, may also play a major role in the pathophysiology of mood dysregulation (Ivleva et al., 2010). Considering the above, *HTR1A* was considered to have an influence in mood regulation. However, we reported that *HTR1A* was associated with methamphetamine-induced psychosis in the Japanese population (Kishi et al., 2009c). We also detected a marginal association between *HTR1A* and schizophrenia in the Japanese population. Considering the neurodevelopmental model of the pathophysiology of both disorders, *HTR1A* may relate to neurodevelopment (Ivleva et al., 2010). It will be necessary to conduct further studies, including environmental factors.

The LD of rs6295 and rs878567 in our BP samples was looser than in controls and schizophrenia samples (r^2 value: controls = 0.160, schizophrenia = 0.101 and BP = 0.00600). Although we detected no association between *HTR1A* and BP in the single-marker association analysis, it may be that the difference in LD reflects the haplotype-wise analysis.

We detected an association between rs6295 and BP in the meta-analysis. The studies of Huang and colleagues and Sullivan and colleagues found no association between rs6295 in *HTR1A* and BP (Huang et al., 2004; Sullivan et al., 2009). However, in our meta-analysis, we detected an association between *HTR1A* and BP. The following may be causes for these different results: First, because the samples in the two original studies were small, there is a possibility of type II errors in their studies. Second, although we did not detect significant heterogeneity among ORs ($P(Q) = 0.789$), the MAFs of the studies included in the meta-analysis were each different. Third, because there are few samples to use in meta-analysis, the significant associations between *HTR1A* and BP in the case-control study and the meta-analysis also may have been due to type I errors. Further, different screening methods were used in each study for the samples included in this meta-analysis. Rs 6295 is associated with disorders, including major depressive disorder (Lemonde et al., 2003; Parsey et al., 2006; Anttila et al., 2007; Kraus et al., 2007; Neff et al., 2009),

Table 3
Haplotype-wise analysis of *HTR1A*.

Haplotype rs6295–rs878567	Phenotype	Number of subjects	Individual haplotype frequency	OR[a]	95% CI[b]	Individual *P*-value[c]	Phenotype	Global *P*-value[c]	Corrected Global *P*-value[c,d]
C-C (reference)	Controls	1372	0.814						
	Schizophrenia	635	0.788	1.20	0.971–1.47	0.0921			
	Bipolar disorder	736	0.761	1.38	1.19–1.59	**0.0000176**	Schizophrenia	0.0935	
G-C	Controls	314	0.186				Bipolar disorder	**0.0000203**	**0.000244**
	Schizophrenia	171	0.212	1.20	0.971–1.47	0.0921			
	Bipolar disorder	231	0.239	1.38	1.19–1.59	**0.0000176**			

Adjustment for age and gender.
[a] OR: odds ratio.
[b] CI: confidence interval.
[c] Bold numbers represent significant *P*-value.
[d] Calculated by Bonferroni correction (12 times).

Table 4
Studies included in meta-analysis for rs6295.

Author	Yesr	Ethnic	Diagnostic system	N[a]			C allele[a]			G allele[a]		
				SCZ	BP	CON	SCZ	BP	CON	SCZ	BP	CON
Kishi		Asian	DSM-IV	857	1028	1810	1036	1475	2726	392	581	894
Huang	2004	Caucasian	DSM-IV	108	88	107	86	81	118	130	95	96
Sullivan	2009	Caucasian	DSM-IV	-	32	47	-	31	57	-	31	35

a BP: bipolar disorder SCZ: schizophrenia CON: controls.

panic disorder (Strobel et al., 2003) and antidepressant response in major depressive disorder (MDD) (Lemonde et al., 2004; Serretti et al., 2004; Arias et al., 2005; Hong et al., 2006; Parsey et al., 2006; Yu et al., 2006). Recently, we reported that *HTR1A* was associated with MDD in a meta-analysis (Kishi et al., 2009b). In addition, we reported that *HTR1A* was associated with Japanese methamphetamine-induced psychosis patients (Kishi et al., 2009c). Our previous studies selected the same variant as this study. Huang and colleagues reported that rs6295 was associated with schizophrenia (Huang et al., 2004). However, we could not replicate the association between rs6295 and schizophrenia found in their study (Huang et al., 2004). We also found no association between rs6295 and schizophrenia in the meta-analysis. Recent studies reported that rs6295 was associated with improvement in negative symptoms from antipsychotics such as risperidone (Reynolds et al., 2006; Wang et al., 2008; Mossner et al., 2009) and that 5-HT1A receptor agonists such as tandospirone produced improvements in cognitive impairment in schizophrenia (Sumiyoshi et al., 2001a, 2007; Meltzer and Sumiyoshi, 2008). We assume that quantitative traits, including negative symptoms and cognitive symptoms for schizophrenic patients, will be key features in assessing the genetic contribution of *HTR1A* to schizophrenia.

The heterogeneity in this meta-analysis for schizophrenia may have resulted from: (1) different ancestries (Asian population vs. Caucasian population), (2) the small size of the overall sample included in the meta-analysis (851 schizophrenic patients and 911 control subjects) and (3) the inclusion of different samples in the screening method in the meta-analysis (Shi et al., 2008).

A few points of caution should be mentioned with respect to our results. First, our results in the case-control study and meta-analysis may be due to biased samples, such as unmatched age and gender samples, or to small sample sizes such as case-control genetic association studies (Shi et al., 2009). Therefore, the significant associations between *HTR1A* and BP in the case-control study and meta-analysis may be due to type I errors. However, we performed a logistic regression analysis to compare the phenotypes of each of the examined SNP genotypes, using several clinical factors as other independent variables to adjust for possible confounding. Second, we did not perform a mutation scan of *HTR1A*. Because we consider it to be difficult to evaluate the association of such extremely rare variants from the viewpoint of statistical power, a replication study using a larger sample is required for conclusive results. Third, we did not include GWAS data regarding rs6295 in this meta-analysis. Fourth, we combined Asian and Caucasian populations in this meta-analysis. However, we did not detect significant heterogeneity for BP samples. In addition, we included the different samples of the screening method in the meta-analysis.

5. Conclusion

In conclusion, our results suggest that *HTR1A* may play a role in the pathophysiology of Japanese BP, but not Japanese schizophrenia. Further, in the meta-analysis, *HTR1A* was associated with BP patients.

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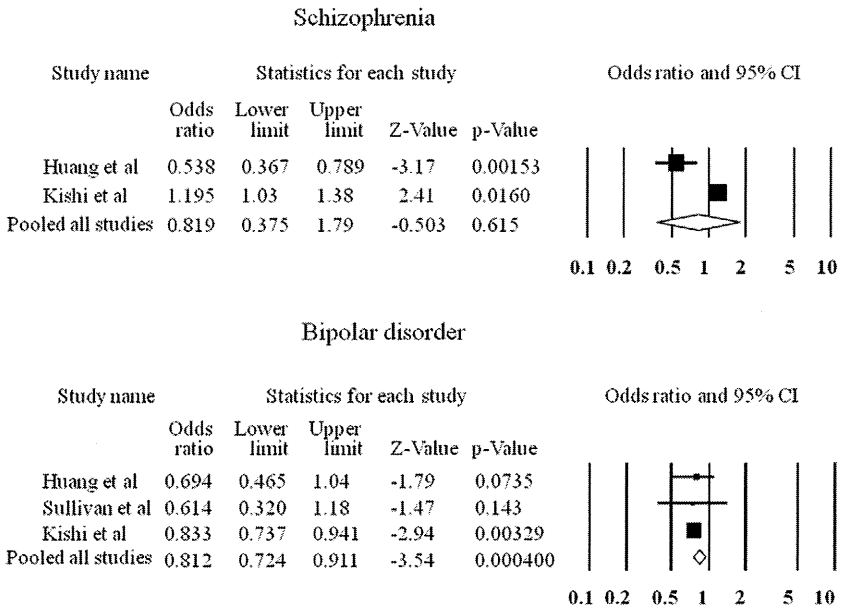

Fig. 1. Forest plots of OR with 95% CI for rs6295.

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Full length article

Serotonin 6 receptor gene is associated with methamphetamine-induced psychosis in a Japanese population

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ABSTRACT

Background: Altered serotonergic neural transmission is hypothesized to be a susceptibility factor for psychotic disorders such as schizophrenia. The serotonin 6 (5-HT6) receptor is therapeutically targeted by several second generation antipsychotics, such as clozapine and olanzapine, and D-amphetamine-induced hyperactivity in rats is corrected with the use of a selective 5-HT6 receptor antagonist. In addition, the disrupted prepulse inhibition induced by D-amphetamine or phencyclidine was restored by 5-HT6 receptor antagonist in an animal study using rats. These animal models were considered to reflect the positive symptoms of schizophrenia, and the above evidence suggests that altered 5-HT6 receptors are involved in the pathophysiology of psychotic disorders. The symptoms of methamphetamine (METH)-induced psychosis are similar to those of paranoid type schizophrenia. Therefore, we conducted an analysis of the association of the 5-HT6 gene (*HTR6*) with METH-induced psychosis.
Method: Using five tagging SNPs (rs6693503, rs1805054, rs4912138, rs3790757 and rs9659997), we conducted a genetic association analysis of case–control samples (197 METH-induced psychosis patients and 337 controls) in the Japanese population. The age and sex of the control subjects did not differ from those of the methamphetamine dependence patients.
Results: rs6693503 was associated with METH-induced psychosis patients in the allele/genotype-wise analysis. Moreover, this association remained significant after Bonferroni correction. In the haplotype-wise analysis, we detected an association between two markers (rs6693503 and rs1805054) and three markers (rs6693503, rs1805054 and rs4912138) in *HTR6* and METH-induced psychosis patients, respectively.
Conclusion: *HTR6* may play an important role in the pathophysiology of METH-induced psychosis in the Japanese population.

1. Introduction

Abnormalities in serotonergic neural transmission are hypothesized to be a susceptibility factor for psychotic disorders, including schizophrenia and drug-related conditions such as methamphetamine (METH)-induced psychosis (Geyer and Vollenweider, 2008; Meltzer et al., 2003). Recently, we reported that the serotonin 1A receptor gene (*HTR1A*) was associated with Japanese METH-induced psychosis patients (Kishi et al., 2010a). The serotonin 6 (5-HT6) receptor is targeted by several second generation antipsychotics, such as clozapine and olanzapine (Bymaster et al., 2001). Therefore, the 5-HT6 receptor is known as a therapeutic target in psychotic disorders. The 5-HT6 receptor is expressed in the frontal cortex, hippocampus, amygdala and striatum, where it is prevalent on gamma-amino butyric acid (GABA)-ergic neurones and activation indirectly regulates a variety of neurotransmitters,

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including serotonin (5-HT), acetylcholine, glutamate and dopamine (Chalmers and Watson, 1991; Fone, 2008; King et al., 2008). 5-HT6 receptor antagonist was shown to produce an increase in amphetamine-induced locomotor activity and also to augment amphetamine-induced increases in extracellular dopamine within the rat prefrontal cortex (Frantz et al., 2002). Also, 5-HT6 antisense injections in the rat brain decreased 5-HT release in the prefrontal cortex (Yoshioka et al., 1998a,b). In addition, 5-HT6 receptor antagonist has been reported to enhance glutamatergic, noradrenergic, and dopaminergic neurotransmission in the rat striatal and frontal cortex (Dawson et al., 2000). These altered neurotransmitters suggest that 5-HT6 might be involved in the pathophysiology of psychotic disorders (Lang et al., 2007). D-amphetamine-induced hyperactivity in rats was corrected with the use of a selective 5-HT6 receptor antagonist. In addition, disrupted prepulse inhibition induced by D-amphetamine or phencyclidine (PCP) in rats was restored by 5-HT6 receptor antagonist. These animal models were considered to reflect the positive symptoms of schizophrenia. Dawson and colleagues also suggested that the 5-HT6 receptor has a regulatory function on several neurotransmitters, when dopaminergic neurotransmission in the rat brain is enhanced by amphetamine. Patients with schizophrenia show cognitive deficits in verbal memory, working memory, attention, processing speed and other areas. Several studies have reported that the influence on cognitive function differs with the kind of antipsychotic (Carter et al., 2008; Crespo-Facorro et al., 2009; Cuesta et al., 2009; Davidson et al., 2009; Keefe et al., 2006; Lindenmayer et al., 2007; Purdon et al., 2000, 2003; Riedel et al., 2007; Woodward et al., 2005). Rodefer and colleagues reported that abnormalities in executive function induced by PCP in rats were restored by the 5-HT6 receptor antagonist SB 271046 but not risperidone, clozapine, olanzapine, or the 5-HT2A receptor antagonist M100907. 5-HT6 receptors are located primarily in the striatum, olfactory tubercles, nucleus accumbens and hippocampus. It is known that functions of serotonin 6 receptors include modulation of cholinergic and dopaminergic neurotransmission. They suggested that the combination of 5-HT6 antagonistic activity and the absence of antimuscarinic activity may play an important role in the pharmacological profile for improved antipsychotic drugs for cognitive dysfunction in schizophrenia. Tsai et al. reported that the 5-HT6 receptor gene (*HTR6*) was associated with schizophrenia in the Chinese population. These facts suggest a crucial relationship between the 5-HT6 receptor and schizophrenia, and that *HTR6* is an adequate candidate for the etiology of schizophrenia. *HTR6* (OMIM * 601109, 2 exons in this genomic region spanning 15.076 kb) is located on 1p36-35.

The symptoms of METH-induced psychosis are similar to those of paranoid type schizophrenia (Sato et al., 1992), and it may be that METH-induced psychosis and schizophrenia have common susceptibility genes (Bousman et al., 2009). In support of this hypothesis, we reported that the V-act murine thymoma viral oncogene homologue 1 (AKT1) gene was associated with METH-induced psychosis (Ikeda et al., 2006) and schizophrenia (Ikeda et al., 2004) in the Japanese population. Therefore, we conducted an analysis of the association of *HTR6* with METH-induced psychosis, using the recently recommended strategy of 'gene-based' association analysis (Neale and Sham, 2004).

2. Materials and methods

2.1. Subjects

The subjects in the association analysis were 197 METH-induced psychosis patients (164 males (83.2%) and 33 females (16.8%); mean age ± standard deviation (SD) 37.6 ± 12.2 years) and 337 healthy controls (271 males (80.4%) and 66 females (19.6%); 37.6 ± 14.3 years). The age and sex of the control subjects did not differ from those of the methamphetamine dependence patients. All subjects were unrelated to each other, ethnically Japanese, and lived in the central area of Japan. All the patients examined in this study suffered not only from METH-induced psychosis (ICD-10-DCR criteria (F15.5)) but also METH dependence (ICD-10-DCR criteria (F15.2)). Consensus diagnoses of METH-induced psychosis were made by the trained psychiatrists according to the ICD-10-DCR criteria (F15.5) on the basis of interviews and medical records. The patients with methamphetamine psychosis in the present study usually showed predominant positive symptoms such as delusion and hallucination. We excluded cases in which the predominant symptoms were of the negative and/or disorganized type in order to maintain the homogeneity of the patient group. The patients were categorized by prognosis into two types, a transient type and a prolonged type, based on the duration of the psychotic state after METH discontinuance. The transient type of patient was defined as a patient whose symptoms improved within 1 month after METH discontinuance and the start of treatment with antipsychotic, and the prolonged type was defined as a patient whose psychosis continued for more than 1 month after METH discontinuance and the start of treatment with an antipsychotic. In this study, there were 112 patients (56.9%) with the transient type and 85 patients (43.1%) with the prolonged type patients of METH psychosis. Cannabinoids were the most frequency abused drugs (31.4%), followed by cocaine (9.09%), LSD (9.09%), opioids (7.69%), and hypnotics (7.69%). Subjects with METH use disorder were excluded if they had a clinical diagnosis of psychotic disorder, mood disorder, anxiety disorder, or eating disorder. More detailed characterizations of these subjects have been published elsewhere (Kishi et al., 2008a, 2010a). All healthy controls were also psychiatrically screened based on unstructured interviews including current and past psychiatric history. None had severe medical complications such as liver cirrhosis, renal failure, heart failure or other Axis-I disorders according to ICD-10DCR.

The study was described to subjects and written informed consent was obtained from each. This study was approved by the Ethics Committees at Fujita Health University and the Nagoya University School of Medicine, and by each participating member of the Institute of the Japanese Genetics Initiative for Drug Abuse (JGIDA).

2.2. SNPs selection and linkage disequilibrium (LD) evaluation

We first consulted the HapMap database (release#27. February 2009, www.hapmap.org, population: Japanese Tokyo: minor allele frequencies (MAFs) of more than 0.05) and included 10 SNPs covering *HTR6* (5′-flanking regions including about 4 kb from the initial exon and about 2 kb downstream (3′) from the last exon: HapMap database contig number Chr1:19,864,367.19878641). Then five tagging SNPs (rs6693503, rs1805054, rs4912138, rs3790757 and rs9659997) were selected with the criteria of an 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) of the HAPLOVIEW software (Barrett et al., 2005). These 5 tagging SNPs were then used for the following association analysis.

2.3. SNPs genotyping

We used TaqMan assays (Applied Biosystems, Inc., Foster City, CA,) for both SNPs. Detailed information, including primer sequences and reaction conditions, is available on request.

2.4. Statistical analysis

Genotype deviation from the Hardy–Weinberg equilibrium (HWE) was evaluated by Chi-square test (SAS/Genetics, release 8.2, SAS Japan Inc., Tokyo, Japan).

Marker-trait association analysis was used to evaluate allele- and genotype-wise associations with the Chi-square test (SAS/Genetics, release 8.2, SAS Japan Inc., Tokyo, Japan), and a haplotype-wise association analysis was conducted with a likelihood ratio test using the COCAPHASE2.403 program (Dudbridge, 2003). We used the permutation test option as provided in the haplotype-wise analysis to avoid spurious results and correct for multiple testing. Permutation test correction was performed using 10,000 iterations (random permutations). In addition, Bonferroni's correction was used to control inflation of the type I error rate in the single marker association analysis and in the individual haplotype-wise analysis. We had already performed a permutation test for global *P*-value in the haplotype-wise analysis. We also performed an explorative analysis of subjects divided by clinical diagnosis (transient type vs prolonged type). The significance level for all statistical tests was 0.05.

3. Results

The result of LD evaluation using our control samples can be seen Fig. 1. However, the LD structure was not tight in any of the tagging SNPs in our control samples. Genotype frequencies of all SNPs were in HWE (Table 1). Rs6693503 was associated with METH-induced psychosis patients in the allele/genotype-wise analysis (P allele = 0.00228 and P genotype = 0.000214) (Table 1). Moreover, the significance of these associations remained after Bonferroni correction (P allele = 0.00107 and P genotype = 0.0114) (Table 1). In addition, we detected an association

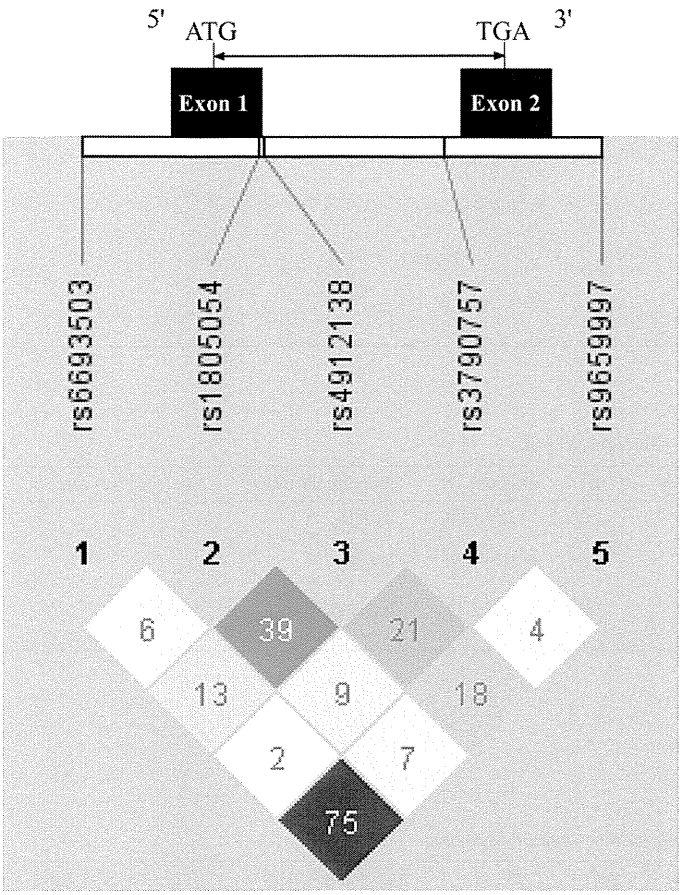

Fig. 1. LD evaluation and tagging SNPs in *HTR6*. ATG is the start codon and TGA is the stop codon. Vertical bars represent exons. Color scheme is based on r^2 value. Other information can be seen at the Haploview website.

between rs6693503 and rs1805054 in *HTR6* and METH-induced psychosis patients in the haplotype-wise analysis ($P=0.0314$) (Table 2). Rs6693503–rs1805054–rs4912138 was associated with METH-induced psychosis patients in the haplotype-wise analysis ($P<0.0001$) (Table 2). Haplotype analysis for rs6693503 and rs1805054 indicated three common haplotypes (A–C, A–T and G–C). Among them, the G–C haplotype was highly prevalent in subjects with METH-induced psychosis (corrected P-value = 0.0279) (Table 3). Haplotype analysis for rs6693503, rs1805054 and rs4912138 also indicated five common haplotypes (A–C–A, A–C–G, A–T–G, G–C–A and G–C–G). Among them, the G–C–A haplotype was highly prevalent in subjects with METH-induced psychosis (corrected P-value <0.0001) (Table 4).

Subcategory analyses were conducted on a clinical parameter (prognosis of psychosis) (Table 5). rs3790757 was associated with prognosis of psychosis in METH-induced psychosis patients in the allele-wise analysis (Table 5). However, the significance of this association disappeared after Bonferroni correction (Table 5). We also found no association between *HTR6* and the clinical features of patients with METH psychosis in the haplotype-wise analysis (Table 6).

4. Discussion

We found associations between rs6693503 in *HTR6*, which is located in a promoter region, and Japanese METH-induced psychosis patients in the allele/genotype-wise analysis. Therefore, we considered that the association between *HTR6* haplotype and METH-induced psychosis patients in this study might be reflected in rs6693503, and reasoned that *HTR6* may play an important role in the pathophysiology of METH-induced psychosis in the Japanese population. However, our samples are small. Although Bonferroni's correction was used to control inflation of the type I error rate, there is a possibility of type I error in these results.

Recently, we reported that *HTR1A* was associated with METH-induced psychosis in the Japanese population. To evaluate the interactions with each SNP in *HTR1A* and *HTR6* (197 METH-induced psychosis patients and 337 controls), we 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 METH-induced psychosis (data not shown).

Several animal studies reported that the 5-HT6 receptor antagonist restored behavior that had become abnormal as a result of amphetamine (Dawson et al., 2000; Frantz et al., 2002; Yoshioka et al., 1998a,b). These findings suggest that altered serotonergic neural transmission caused by abnormalities in 5-HT6 receptors may be involved in the development of METH-induced psychosis. However, all the patients examined in this study suffered not only from METH-induced psychosis but also METH dependence. METH stimulates the release of dopamine in the mesolimbic system (Munzar et al., 2004), and dopamine is in turn involved in the reinforcing action of many addictive drugs such as METH (Vocci et al., 2005). The reciprocal action of these mechanisms may contribute to METH

Table 1
Association analysis of *HTR6* with methamphetamine-induced psychosis.

SNP ID[a]	Phenotype[b]	MAF[c]	*N*	Genotype distribution[d]			*P*-value[e,f]			Corrected *P*-value[f,g]	
				M/M	M/m	m/m	HWE	Genotype	Allele	Genotype	Allele
rs6693503	Control	0.134	337	257	70	10	0.0602				
A>G	METH-induced psychosis	0.221	197	124	59	14	0.0688	**0.00228**	**0.000214**	**0.0114**	**0.00107**
rs1805054	Control	0.292	337	162	153	22	0.0738				
C>T	METH-induced psychosis	0.309	197	90	92	15	0.195	0.816	0.550		
rs4912138	Control	0.493	337	87	170	80	0.864				
A>G	METH-induced psychosis	0.484	197	55	93	49	0.441	0.764	0.879		
rs3790757	Control	0.191	337	220	105	12	0.903				
C>T	METH-induced psychosis	0.238	197	115	70	12	0.758	0.179	0.0672		
rs9659997	Control	0.157	337	243	82	12	0.132				
C>T	METH-induced psychosis	0.144	197	146	45	6	0.280	0.868	0.581		

[a] Major allele > minor allele.
[b] METH-induced psychosis: methamphetamine-induced psychosis patients.
[c] MAF, minor allele frequency.
[d] M, major allele; m, minor allele.
[e] HWE, Hardy–Weinberg equilibrium.
[f] Bold numbers represent significant *P*-value.
[g] Calculated by Bonferroni correction (5 tests).

Table 2
Haplotype-wise analysis between *HTR6* and methamphetamine-induced psychosis.

	Global *P*-value[a]			
	2 window	3 window	4 window	5 window
rs6693503				
	0.0314			
rs1805054		**<0.0001**		
	0.531		0.141	
rs4912138		0.203		0.138
	0.167		0.328	
rs3790757		0.201		
	0.180			
rs9659997				

[a] Bold numbers represent significant global *P*-value.

Table 3
Haplotype-wise analysis between rs6693503–rsl 805054 in HTR6 and methamphetamine-induced psychosis.

rs6693503–rsl805054	Phenotype[a]	Individual haplotype frequency	OR[b]	95% CI[c]	Individual *P*-value[d]	Corrected *P*-value[d,e]
A–C	Control	0.574	1.00	1.00–1.00	0.0824	
	METH-induced psychosis	0.519				
A–T	Control	0.292	1.09	0.813–1.46	0.863	
	METH-induced psychosis	0.287				
G–C	Control	0.134	1.61	1.13–2.29	**0.00931**	**0.0279**
	METH-induced psychosis	0.194				

[a] METH-induced psychosis: methamphetamine-induced psychosis patients.
[b] OR, odds ratio.
[c] 95% CI, 95% confidence interval.
[d] Bold numbers represent significant *P*-value.
[e] Calculated by Bonferroni's correction (3 tests).

Table 4
Haplotype-wise analysis between rs6693503–rsl805054–rs4912138 in HTR6 and methamphetamine-induced psychosis.

rs6693503–rsl805054–rs4912138	Phenotype[a]	Individual haplotype frequency	OR[b]	95% CI[c]	Individual *P*-value[d]	Corrected *P*-value[d,e]
A–C–A	Control	0.488	1.00	1.00–1.00	0.227	
	METH-induced psychosis	0.444				
A–C–G	Control	0.0862	0.948	0.574–1.57	0.351	
	METH-induced psychosis	0.0744				
A–T–G	Control	0.292	1.07	0.788–1.44	0.0772	
	METH-induced psychosis	0.284				
G–C–A	Control	0.00161	54.6	7.36–405	**<0.0001**	**<0.0001**
	METH-induced psychosis	0.0798				
G–C–G	Control	0.132	0.978	0.649–1.47	0.646	
	METH-induced psychosis	0.118				

[a] METH-induced psychosis: methamphetamine-induced psychosis patients.
[b] OR, odds ratio.
[c] 95% CI, 95% confidence interval.
[d] Bold numbers represent significant *P*-value.
[e] Calculated by Bonferroni's correction (5 tests).

Table 5
Association between HTR6 and clinically subcategorized METH-induced psychosis.

SNP ID[a]	Phenotype	MAF[b]	N	Genotype distribution[c]			*P*-value[d,e]			Corrected *P*-value[f]
				MM	M/m	m/m	HWE	Genotype	Allele	Allele
rs6693503	Transient type	0.223	112	70	34	8	0.187			
A > G	Prolonged type	0.217	85	54	25	6	0.209	0.989	0.895	
rs1805054	Transient type	0.299	112	52	5338	7	0.173			
C>T	Prolonged type	0.324	85	38	39	8	0.657	0.709	0.604	
rs4912138	Transient type	0.469	112	31	57	24	0.817			
A>G	Prolonged type	0.506	85	24	36	25	0.159	0.370	0.465	
rs3790757	Transient type	0.281	112	59	43	10	0.594			
C>T	Prolonged type	0.182	85	56	27	2	0.547	0.0650	**0.0225**	0.113
rs9659997	Transient type	0.156	112	80	29	3	0.849			
C>T	Prolonged type	0.129	85	66	16	3	0.129	0.491	0.453	

[a] Major allele > minor allele.
[b] MAF, minor allele frequency.
[c] M, major allele; m, minor allele.
[d] HWE, Hardy–Weinberg equilibrium.
[e] Bold numbers represent significant *P*-value.
[f] Calculated by Bonferroni correction (5 tests).

Table 6
Association between *HTR6* and clinically subcategorized METH-induced psychosis (transient type vs prolonged type).

	Global *P*-value			
	2 window	3 window	4 window	5 window
rs6693503				
	0.796			
rs1805054		0.942		
	0.756		0.426	
rs4912138		0.104		0.259
	0.0709		0.0918	
rs3790757		0.0783		
	0.0669			
rs9659997				

dependence. In addition, increased dopamine in the mesolimbic system is considered to produce psychotic symptoms such as hallucinations and delusions (Laviolette, 2007). Therefore, patients with METH dependence may develop psychotic symptoms in the long term revealed in the long term may cause the psychotic symptoms.

We reported that the AKT1 gene was associated with METH-induced psychosis (Ikeda et al., 2006) and schizophrenia (Ikeda et al., 2004) in the Japanese population. These findings may indicate that METH-induced psychosis and schizophrenia have common susceptibility genes (Bousman et al., 2009). In this study, we found an association between *HTR6* was associated with METH-induced psychosis in the Japanese population. Tsai et al. reported that *HTR6* was associated with schizophrenia in the Chinese population (Tsai et al., 1999a). However, other studies, including two studies using Japanese samples, showed no association (Chiu et al., 2001; Ohmori et al., 2001; Shinkai et al., 1999; Vogt et al., 2000). Past these association studies using the "candidate gene approach" had the serious problem of small samples (Chiu et al., 2001; Ohmori et al., 2001; Shinkai et al., 1999; Tsai et al., 1999a; Vogt et al., 2000). Therefore, a replication study of the association between *HTR6* and schizophrenia will be needed using the recently recommended strategy of 'gene-based' association analysis including rs1805054 (C267T) (Neale and Sham, 2004), and larger samples than the original studies (Chiu et al., 2001; Shinkai et al., 1999; Tsai et al., 1999a; Vogt et al., 2000).

Rs1805054 (C267T) is a silent mutation, which does not cause a change in the amino acid sequence (Kohen et al., 1996). Several studies have suggested that it may affect translation through the secondary structure and stability of the mRNA, or be in LD with a functional variant. Therefore, genetic association studies of psychiatric disorders such as schizophrenia (Chiu et al., 2001; Shinkai et al., 1999; Tsai et al., 1999a; Vogt et al., 2000), bipolar disorder (Hong et al., 1999), major depressive disorder (MDD) (Hong et al., 1999) and Alzheimer's disease (Alvarez-Alvarez et al., 2003; Kan et al., 2004; Liu et al., 2001; Tsai et al., 1999b), and pharmacogenic studies of the antipsychotic response in schizophrenia (Ikeda et al., 2008) have so far investigated only rs1805054 in *HTR6*. However, we found an association of rs6693503 but not rs1805054 (C267T) with METH-induced psychosis patients in this single marker association study.

Rs6693503 is located in the promoter region in *HTR6*, and two common haplotypes (rs6693503–rs1805054: G–C and rs6693503–rs1805054–rs4912138: G–C–A) were associated with METH-induced psychosis patients. Because of the possibility that rs6693503 or these haplotypes influence biological function in the brain, we suggest that functional analysis of these variants should be performed in future studies.

Patients with schizophrenia show cognitive deficits in verbal memory, working memory, attention, processing speed and other areas (Green, 1996). Cognitive impairment in these patients is significantly associated with insight, social skills, and delay in returning to normal life (Monteiro et al., 2008). Abnormalities in cognitive function are considered to be endophenotypes for schizophrenia (Walters and Owen, 2007). Such impairments may be partially mediated by serotonin receptors, including the 5-HT6 receptor. Several genetic studies reported that the *HTR6* was associated with Alzheimer's disease. Therefore, investigations on the relationship between cognitive function in schizophrenia and variants of *HTR6* are needed.

A few points of caution should be mentioned with respect to our results. Firstly, the positive association may be due to small sample size. Ideal samples for this study are METH use disorder samples with and without psychosis. Because we had only a few METH use disorder samples without psychosis, and we wanted to avoid statistical error, we did not perform an association analysis with these samples. Secondly, we did not include a mutation scan to detect rare variants. We designed the study based on the common disease-common variants hypothesis (Chakravarti, 1999). However, Weickert and colleagues have shown associations between a common disease such as schizophrenia and rare variants (Weickert et al., 2008). If the genetic background of METH-induced psychosis is described by the common disease-rare variants hypothesis, further investigation such as medical resequencing using larger samples will be required. However, statistical power is needed to evaluate the association of rare variants. Lastly, our subjects did not undergo structured interviews. However, in this study patients were carefully diagnosed according to DSM-IV criteria with consensus of at least two experienced psychiatrists on the basis of a review of medical records (Kishi et al., 2008a,b, 2009, 2010a,b). In addition, when we found misdiagnosis in a patient, we promptly excluded the misdiagnosed case to maintain the precision of our sample. To overcome these limitations, a replication study using larger samples or samples of other populations will be required for conclusive results.

In conclusion, our results suggest that *HTR6* may play a major role in the pathophysiology of METH-induced psychosis in the Japanese population. However, because we did not perform a mutation scan of *HTR6*, a replication study using a larger sample may be required for conclusive results.

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Conflict of interest

All authors declare that they have no conflict of interest.

Contributors

All authors contributed to and have approved the final manuscript.

Ethical approval

Written informed consent was obtained from all subjects. This study was approved by the ethics committees at Fujita Health University, Nagoya University Graduate School of Medicine and each participating member of the Institute of the Japanese Genetics Initiative for Drug Abuse (JGIDA).

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Gene-wide association study between the methylenetetrahydrofolate reductase gene (*MTHFR*) and schizophrenia in the Japanese population, with an updated meta-analysis on currently available data

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ABSTRACT

Methylenetetrahydrofolate reductase (MTHFR) is a critical molecule for single-carbon transfer reactions. Recent evidence suggests that polymorphisms of MTHFR are related to neural tube deficits and the pathogenesis of schizophrenia. While several studies have demonstrated associations between the gene encoding the MTHFR (*MTHFR*) polymorphisms and schizophrenia, these studies lack consistency. Therefore, we conducted a gene-wide association study (patients with schizophrenia = 696, control subjects = 747) and performed imputation analysis. Additionally, we performed meta-analysis on currently available data from 18 studies for two common functional polymorphisms (rs1801131 and rs1801133).

There were no significant associations with schizophrenia in the single marker analysis for the seven tagging SNPs of *MTHFR*. In the haplotypic analysis, a nominally significant association was observed between the haplotypes, which included four SNPs (rs1801133, rs17421511, rs17037396, and rs9651118) and the schizophrenic patients. Additionally, the imputation analysis demonstrated there were several associated markers on the *MTHFR* chromosomal region. However, confirmatory analyses of three tagging SNPs (rs1801133, rs17037396, and rs9651118) and the top SNP (rs17421511) for the imputation results (patients with schizophrenia = 797, control subjects = 1025) failed to replicate the haplotypic analysis and the imputation results. These findings suggest that *MTHFR* polymorphisms are unlikely to be related to the development of schizophrenia in the Japanese population. However, since our meta-analysis results demonstrated strong support for association of rs1801133 with schizophrenia, further replication studies based on a gene-wide approach need to be considered.

1. Introduction

Schizophrenia is a chronic and disabling mental disorder with a lifetime prevalence of approximately 1% in the global population (Freedman, 2003). Accumulating evidence suggests that both genetic and environmental factors contribute to the

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etiology of schizophrenia (Burmeister et al., 2008). Although schizophrenia has a high heritability with rates estimated at 80% (Sullivan et al., 2003), there has been no consistent replication found for the schizophrenia candidate genes (Harrison and Weinberger, 2005). Recent genome-wide association (GWA) studies have demonstrated new promising susceptibility genes for schizophrenia (O'Donovan et al., 2008), as well as for other common diseases (Rioux et al., 2007; The Wellcome Trust Case Control Consortium, 2007; Zeggini et al., 2007). Therefore, use of this methodology can be advantageous when trying to detect potential genetic factors responsible for the development of these disorders. In addition, by focusing on the specific molecular pathway related to the pathophysiology of schizophrenia, this may also be useful when trying to identify susceptibility genes that have a mild contribution to the development of the disease (Kirov et al., 2005).

Dysfunction of homocysteine metabolism has been linked to neurodevelopmental disorders, including neural tube defects (NTDs) (Blom et al., 2006; van der Put et al., 1995), schizophrenia (Allen et al., 2008; Muntjewerff et al., 2006), and depression (Lewis et al., 2006), in addition to other diseases and syndromes (Hobbs et al., 2000; Kluijtmans et al., 1996; Qian et al., 2007). Recent studies have also suggested that elevated plasma homocysteine levels are observed in major psychiatric disorders such as schizophrenia and bipolar disorder (Levine et al., 2005). Plasma homocysteine levels affect the intracellular methylation process of DNA, lipids, proteins, and neurotransmitters (Scott and Weir, 1998). Both elevated homocysteine levels along with physiological levels of its oxidized derivatives, such as homocysteic acid and homocysteine sulfinic acid, have been shown to be toxic for neurons and vascular endothelial cells (Zou and Banerjee, 2005). While levels of homocysteine are affected by various genes involved in the homocysteine metabolic pathway and by environmental factors such as folate or vitamin B_{12} intake (Refsum et al., 2004), methylenetetrahydrofolate reductase (MTHFR) also plays a major role in this pathway. MTHFR converts 5,10-methylenetetrahydrofolate to 5-methyltetrahydrofolate, which serves as a carbon donor for the methylation of homocysteine, leading to the generation of S-adenosylmethionine (SAM) (Andreoli and Maffei, 1975). SAM is a major source of methyl groups in the brain (Godfrey et al., 1990) and is involved in catechol-O-methyltransferase (COMT) reactions such as the catabolism of serotonin and other catecholamines (Anguelova et al., 2003; Chen et al., 2004). Freeman et al. (1975) reported there is direct evidence linking decreased MTHFR activity to schizophrenia (Freeman et al., 1975). These findings have led to multiple genetic analyses examining the link between the MTHFR gene (gene symbol: *MTHFR*, GenBank accession number: NM_005957) and schizophrenia.

MTHFR is composed of twelve exons (Fig. 1) and is localized on chromosome 1p36.3 (Goyette et al., 1994). It has been suggested that this may be a susceptibility locus for schizophrenia, bipolar disorder (Kempisty et al., 2007) and major depressive disorder (McGuffin et al., 2005). Two common functional polymorphisms of *MTHFR*, C677T (rs1801133) and A1298C (rs1801131), are known to cause a decrease of enzyme activity and affect nucleic synthesis and DNA methylation (van der Put et al., 1998). Several studies have confirmed the possible involvement of these SNPs in psychiatric conditions such as schizophrenia (Regland, 2005) and affective disorders (Arinami et al., 1997). Subjects with homozygosity for the 677 T allele have a mild increase in their plasma homocysteine levels, and these subjects have a higher frequency of neural tube deficits and premature cardiovascular disease as compared to other similar genotype carriers (Bakker and Brandjes, 1997; Matsushita et al., 1997). The impact of this polymorphism varies according to environmental factors, such as folate, vitamin B_2 or vitamin B_{12} (Hustad et al., 2000; Refsum et al., 2004; van der Put et al., 1995). Although some studies have reported that carriers of the 677 T allele in *MTHFR* are associated with an increased risk of schizophrenia (Arinami et al., 1997; Muntjewerff et al., 2005; Sazci et al., 2003), others have shown contradictive results (Kunugi et al., 1998; Vilella et al., 2005; Yu et al., 2004). The association of the *MTHFR* C677T variant with schizophrenia may be linked to the excitatory amino acids hypothesis or to decreased plasma concentrations of SAM that have been reported in psychiatric disorders (Andreoli and Maffei, 1975). Another functional polymorphism, A1298C, also has been shown to decrease MTHFR activity, although van der Put et al. (1998) have reported finding no significant effect of this variant on the plasma homocysteine levels.

A recent meta-analysis demonstrated an association between elevated homocysteine levels or carriers of the 677 T allele and an increased risk of developing schizophrenia (Allen et al., 2008; Muntjewerff et al., 2006). It has been suggested that potential associations between genetic variation in folate metabolism and psychiatric disorders could be plausible biological explanations for these disorders (Coppen and Bolander-Gouaille, 2005).

Taken together, *MTHFR* may be related to the development of schizophrenia. Although a number of studies have demonstrated associations between specific polymorphisms of *MTHFR* and schizophrenia, there have been no gene-based analysis studies. Therefore, it is still difficult to interpret these types of studies due to the inconsistent results that have been derived from some of the confounding factors, such as population

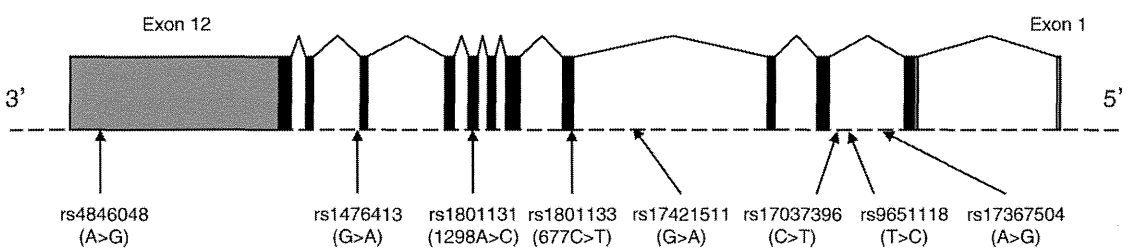

Fig. 1. Genomic structure of *MTHFR*. Black boxes indicate protein-coding regions, while the gray boxes represent the untranslated regions (UTRs). Each box represents *MTHFR* exons. Numbers under the arrows represent the SNP IDs, the tagging SNPs (pairwise tagger: $r^2>0.8$; Haploview 3.32), and the top SNP (rs17421511) of imputation results.

stratifications (ethnic or gender differences) and number of samples. In the present study, we conducted an association study between *MTHFR* and schizophrenia in the Japanese population that was based on the gene-wide approach. In addition, we also performed a meta-analysis on the updated data currently available.

2. Materials and methods

2.1. Subjects

The samples for this association study consisted of 696 patients with schizophrenia and 747 control subjects. The confirmation sample set for four SNPs (rs1801133, rs17421511, rs17037396, and rs9651118), which were positively associated with schizophrenia in the haplotypic analysis and the imputation analysis, consisted of 797 patients with schizophrenia and 1025 control subjects. Detailed demographical data are presented in Supplementary Table 1.

All subjects were unrelated to each other and ethnically Japanese. The schizophrenia diagnosis was made by at least two experienced psychiatrists and based on unstructured patient interviews and reviews of their medical records in accordance with the Diagnostic and Statistical Manual of Mental Disorders, Fourth Edition, Text Revision (DSM-IV-TR) criteria for schizophrenia. All healthy control subjects were also psychiatrically screened on the basis of unstructured interviews.

This study was approved by the Ethics Committee of the Nagoya University Graduate School of Medicine and Fujita Health University. Written informed consent was obtained from each subject.

2.2. Tagging SNP selection

In order to obtain the SNPs that covered the entire coding region as well as the regulatory elements in the 5′ and 3′ flanking areas for both the 1000 base pairs (bps) upstream and downstream of the coding region, we first examined the *MTHFR* genotyping data from the HapMap database (HapMap Data Rel 21/phase II Jan 06, population: Japanese living in Tokyo). Subsequently, the tagging SNPs were selected using the Haploview software version 4.2 in accordance with the criterion of the Tagger program for pairwise tagging, $r^2>0.8$, with minor allele frequency (MAF)>0.1 (de Bakker et al., 2005) (Supplementary Table 2). We excluded rs13306553 due to the unavailability of a reliable genotyping method (genotype call rate<95%). Therefore, a total of seven SNPs were recruited for these genetic association analyses (Fig. 1).

2.3. SNP genotyping

Venous blood was drawn from each subject and genomic DNA was extracted according to standard phenol/chloroform method. SNP genotyping was carried out using the TaqMan allelic discrimination assay (Applied Biosystems, Foster City, CA, USA). TaqMan probes and Universal PCR Master Mix were purchased from Applied Biosystems. Allelic specific fluorescence was measured on the ABI PRISM 7900HT using the Sequence Detection Systems 2.0 software (Applied Biosystems) for allelic discrimination. To exclude low-quality DNA sample or genotyping probes, data sets were filtered on the basis of tagging SNP genotype call rates (95% completeness). Subjects whose percentage of missing genotypes was >10% or who had evidence of possible DNA contamination were excluded from subsequent analyses. For quality control, we randomly selected 10 samples for each SNP and then genotyped these in duplicate in order to evaluate the genotype error rate.

2.4. Imputation and confirmatory association analysis

To estimate genotypes of untyped SNPs located on the analyzed gene region, we conducted an imputation analysis. This method provides enhanced statistical power for the coverage of common variants within the locus of interest. Specifically, based on directly genotyped SNPs and the haplotypes detected in the hapmap JPT sample, a computational algorithm predicted the genotypes at the SNPs that are not directly genotyped in the study sample (Marchini et al., 2007). We carried out this analysis using the MACH 1.0 program (http://www.sph.umich.edu/csg/abecasis/MACH/) in order to calculate the genotypic prediction for the 11 untyped SNPs. These calculations used information from the screening scan for the seven directly typed SNPs and the HapMap database (HapMap Data Rel 21/phase II Jan 06, population: Japanese/Chinese).

The MACH program has been reported to have imputation accuracy rates similar to IMPUTE and both programs are able to outperform fastPHASE, PLINK, and Beagle (Pei et al., 2008). As previously mentioned, the analyzed region of imputation was limited to the *MTHFR* locus. Associated SNPs were pruned based on the linkage disequilibrium (LD) pattern ($r^2>0.8$; Supplementary Table 2) and minor allele frequency (MAF<0.05), with the SNP showing the smallest allelic p value selected for follow up.

2.5. Statistical analysis

Genotype deviation from the Hardy–Weinberg equilibrium (HWE), and marker–trait associations (allelic, genotypic, and haplotypic analysis) were evaluated by using PLINK v1.06 (Purcell et al., 2007). The significance level for all statistical tests was 0.05. Bonferroni correction was used to control inflation of the type I error rate in the allele-wise, genotype-wise, and haplotype-wise analyses. To reduce the total number of tests, clearly unassociated markers were removed in the first stage (screening sample set) of the present study. Conditional on the first stage findings, which used a less stringent nominal level, we subsequently tested the second stage (confirmation sample set) using the augmented data and the data from the first stage. In this joint sample analysis, p values were generated by the Cochran–Mantel–Haenszel stratified analysis, while the Breslow–Day Test was performed for evaluation of heterogeneous associations as implemented in PLINK. Based on the multiplicative model of inheritance, power calculations were performed using the Genetic Power Calculator (Purcell et al., 2003).

2.6. Meta-analysis

We performed a meta-analysis for rs1801131 and rs1801133, which are the two SNPs that have been previously shown to be associated with schizophrenia (Arinami et al., 1997; Betcheva et al., 2009; Feng et al., 2009; Garcia-Miss et al., 2010; Jonsson et

al., 2008; Joober et al., 2000; Kempisty et al., 2007; Kempisty et al., 2006; Kunugi et al., 1998; Lee et al., 2006; Muntjewerff et al., 2005; Philibert et al., 2006; Sazci et al., 2003; Sazci et al., 2005; Tan et al., 2004; Vilella et al., 2005; Yu et al., 2004). Initially, the Q statistic test was performed to assess the heterogeneity in the combined studies. As substantial amounts of variation have been previously observed, we decided to calculate the cumulative odds ratio (OR) and corresponding p value based on a random effect model (OR was calculated based on minor allele observed in Japanese population). Furthermore, use of this calculation was chosen because many investigators consider the random effects model to be a much more natural choice as compared to the fixed effects approach (Ades et al., 2005; DerSimonian and Laird, 1986; Fleiss and Gross, 1991). The significance of the overall OR was determined by the Z-test. Publication bias was assessed using a linear regression analysis to measure funnel plot asymmetry. A probability level of $p<0.05$ was used as the threshold for statistical significance. Comprehensive Meta-Analysis software (Version 2.2.046, Biostat, Englewood, NJ) was used to perform the analysis.

3. Results

Regarding quality control, the genotype calls of the duplicated samples showed complete concordance (data not shown), and all genotype frequencies of the tagging SNPs were consistent with the HWE. There were no significant differences between the schizophrenic patients and the control subjects in both allele and genotype distributions without imputed (untyped) SNP (rs17421511) (Table 1). In the haplotypic analysis, a nominally significant association was observed between the haplotypes including four SNPs (rs1801133, rs17421511, rs17037396, and rs9651118) and schizophrenic patients (Table 1). Imputation analysis showed several associated markers for schizophrenia on the *MTHFR* chromosomal region (Table 2). These nominally significant associations, however, did not survive after Bonferroni correction. After assessment of the HapMap database, the top SNP (rs17421511) was selected to confirm these nominal significant associations between imputed markers and schizophrenia. The results of the genotyping data in confirmatory analyses and joint analyses for the four SNPs (rs1801133, rs17421511, rs17037396, and rs9651118) after Bonferroni correction showed no significant association signal for either the allele and haplotype frequencies with schizophrenic phenotype (Table 3 and Supplementary Table 3). Assuming a multiplicative model of inheritance, a disease prevalence of 1%, and a high LD between the genotyped SNP and risk variant, we obtained more than 80% power in detecting the gene-wide association with schizophrenia when the genotype relative risk was set at 1.28 to 1.38 (screening sample set) and 1.25 to 1.35 (confirmation sample set) (MAF: 0.11 to 0.40 and 0.10 to 0.40, respectively). In the meta-analysis for the two commonly associated SNPs, we used all available data from 18 studies and data from studies that only focused on Asian populations (seven studies) to calculate the cumulative odds ratio (OR). We observed association only at rs1801133 for schizophrenia ($P_{(\text{random model})}=0.000833$), without any population-wise specific effect (Supplementary Tables 4 and 5).

4. Discussion

Even though we applied the gene-based approach in the present study, we could not confirm any significant associations of the *MTHFR* polymorphisms with schizophrenia. In the association analysis, we examined the SNPs covering the entire gene, including all of the tagging SNPs that had at least ~ 10% MAF listed on the HapMap database. For all of the genoõtyped SNPs, there were no associations noted between the patients with schizophrenia and the controls in any of the allele frequencies after Bonferroni correction (Table 1). To confirm our results, we additionally performed an imputation analysis for the estimated untyped SNPs and genotyped three markers (rs1801133, rs17037396, and rs9651118) and the top SNP

Table 1
Results of association analyses (screening sample set).

	dbSNP		Single marker (allele-wise)					Multi marker (haplotype-wise)[a]	
			SCZ[b]	CON[c]	L95[d]	U95[d]	p value	2 markers	3 markers
Maker 1	rs4846048	A>G	0.104	0.107	0.754	1.231	0.767		
								0.878	
Maker 2	rs1476413	G>A	0.203	0.203	0.833	1.210	0.968		0.681
								0.899	
Maker 3	rs1801131	A>C	0.201	0.208	0.796	1.157	0.667		0.801
								0.711	
Maker 4	rs1801133	C>T	0.395	0.404	0.827	1.125	0.643		0.628
								0.034	
Maker 5[e]	rs17421511	G>A	0.174	0.138	1.070	1.624	0.009		0.078
								0.035	
Maker 6	rs17037396	C>T	0.110	0.110	0.789	1.278	0.972		0.052
								0.972	
Maker 7	rs9651118	T>C	0.355	0.350	0.872	1.195	0.794		0.974
								0.902	
Maker 8	rs17367504	A>G	0.111	0.113	0.774	1.249	0.889		

[a]Log likelihood ratio test p value (sliding window analysis with rare haplotype threshold 10%).
[b]SCZ: Schizophrenia.
[c]CON: Control; minor allele frequency.
[d]95% confidence intervals (odds ratio).
[e]Imputed SNP with lowest p value.

Table 2
Allele-wise analysis of imputed SNPs.

dbSNP		MAF[a]	*p* value	Quality[b]
rs17421511	G>A	0.158	0.014	0.907
rs17421560	G>A	0.129	0.544	0.940
rs11121832	C>T	0.144	0.041	0.901
rs2066471	G>A	0.152	0.016	0.920
rs7533315	C>T	0.151	0.016	0.923
rs17037390	G>A	0.122	0.586	0.967
rs17037397	C>A	0.107	0.503	0.998
rs2066470	C>T	0.108	0.499	0.994
rs3753582	T>G	0.108	0.499	0.988
rs13306561	T>C	0.132	0.499	0.937
rs3737965	C>T	0.108	0.499	0.978

[a]MAF: minor allele frequency.
[b]Quality: the average posterior probability for the most likely genotype.

(rs17421511) of imputation results (rs17421511). The nominally significant associations that were detected in haplotype-wise analysis and also in imputation analysis did not survive in confirmatory association analysis (Table 3). Therefore, as previously reported, it is unlikely that other common variants related to schizophrenia are causal to the development of this disease (Chakravarti, 1999).

Several researchers have reported that two common *MTHFR* variants, C677T (rs1801133) and A1298C (rs1801131), are related to the development of schizophrenia (Allen et al., 2008; Gilbody et al., 2007). Even though other investigators could not reproduce these findings (Kunugi et al., 1998; Vilella et al., 2005; Yu et al., 2004), results of a recent meta-analysis support a relationship between the *MTHFR* C677T polymorphism and the risk for schizophrenia (Muntjewerff et al., 2006; van der Put et al., 1995). The 677TT/1298AA (Virgos et al., 1999) and 677CC/1298CC (Sazci et al., 2005) compound genotypes have been shown to be over-represented in schizophrenia samples. These contradictions might be derived from confounding factors such as age, gender, or ethnicity (population stratifications) (Cardon and Palmer, 2003; Munafo and Flint, 2004). The discrepancy between these results and our current results could be due to the locus heterogeneity of this disease. In fact, since the statistical power to detect an association exceeded 80%, there is a low possibility of a type II error. The GRR value that was calculated using the Genetic Power Calculator appeared to be appropriate when compared to promising candidate genes for schizophrenia (Schwab et al., 2003; Shifman et al., 2002). In findings from a recent whole genome association study that focused on schizophrenia (O'Donovan et al., 2008), results suggested that the effect size of common SNPs might be very low, and therefore, sample sizes used for genetic association studies need to be very large. Our current meta-analysis provides indirect support for such a scenario. In order to evaluate the impact of the SNP that was shown to be associated with schizophrenia in our meta-analysis (rs1801133), we have used the PolyPhen-2 (Adzhubei et al., 2010). The software compares the property of the wild-type (ancestral, normal) allele and the corresponding property of the mutant (derived, disease-causing) allele. The alignment pipeline selects a set of homologous sequences using a clustering algorithm and then constructs and refines the multiple alignments. According to the aforementioned calculation, rs1801133 was shown to have a damaging effect on protein structure while the ancestral allele showed the high level of evolutionary conservation (Supplementary Table 6). This finding is consistent with the meta-analysis results, as these demonstrated the associated allele is the risk allele. However, while we could not detect the association in our sample, it is of note that we have detected a publication bias ($t=2.778$, $df=16$, $p=0.013$), and therefore, the pooled *p* value might be overestimated.

In order to be able to elucidate the exact role of genetic variants, definitions of phenotypes are vital for a genetic association study. Therefore, sample stratification using endophenotypes, such as being more specific than phenotypes (e.g., prepulse inhibition, event-related potential, and mismatch negativity), clinical symptoms (e.g., response to medication), or environmental factors (e.g., food intake, supplementation) may be required for these clinical investigations (Braff et al., 2007; Craddock et al., 2006; Gottesman and Gould, 2003). Although we did not take advantage of these types of analytical tests for the genetic association in the present study, these might very well be useful in helping to elucidate the role of *MTHFR* in schizophrenia.

In conclusion, the findings of the present study suggest that *MTHFR* is unlikely to be related to the development of schizophrenia in the Japanese population. However, as our meta-analysis results provided strong support for the association of rs1801133 with schizophrenia, further replication studies based on the gene-wide approach using a large cohort

Table 3
Results of association analyses (confirmation sample set).

dbSNP			Single marker (allele-wise)					Multi marker (haplotype-wise)[a]	
			SCZ[b]	CON[c]	L95[d]	U95[d]	*p* value	2 markers	3 markers
Marker 4	rsl801133	C>T	0.409	0.399	0.910	1.195	0.545		
								0.527	
Marker 5	rs17421511	G>A	0.098	0.098	0.800	1.253	0.991		0.597
								0.924	
Marker 6	rs17037396	T>C	0.104	0.103	0.812	1.258	0.925		0.073
								0.975	
Marker 7	rs9651118	A>G	0.354	0.358	0.856	1.131	0.824		

[a]Log likelihood ratio test *p* value (sliding window analysis with rare haplotype threshold 10%).
[b]SCZ: Schizophrenia.
[c]CON: Control; minor allele frequency.
[d]95% confidence intervals (odds ratio).

of subjects need to be undertaken. In addition, by combining such types of studies with endophenotypes or clinical stratifications, this may provide a better understanding of the pathophysiology of schizophrenia.

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Contributors

Authors Akira Yoshimi, Nagahide Takahashi, and Toshiya Inada designed the study and wrote the protocol. Authors Akira Yoshimi and Yukiko Kawamura conducted SNPs genotyping and statistical analyses. Authors Norio Ozaki, Yukihiro Noda, and Kiyofumi Yamada managed the literature searches and analyses. Author Akira Yoshimi wrote the first draft of the manuscript and Branko Aleksic revised. All authors contributed to and have approved the final manuscript.

Conflict of interest

The authors have no conflicts to declare.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.schres.2010.07.011.

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SHORT COMMUNICATION

An association study between the dymeclin gene and schizophrenia in the Japanese population

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Many gene variants are involved in the susceptibility to schizophrenia and some of them are expected to be associated with other human characters. Recently reported meta-analysis of genetic associations revealed nucleotide variants in synaptic vesicular transport/Golgi apparatus genes with schizophrenia. In this study, we selected the dymeclin gene (*DYM*) as a candidate gene for schizophrenia. The *DYM* gene encodes dymeclin that has been identified to be associated with the Golgi apparatus and with transitional vesicles of the reticulum–Golgi interface. A three-step case–control study of total of 2105 Japanese cases of schizophrenia and 2087 Japanese control subjects was carried out for tag single-nucleotide polymorphisms (SNPs) in the *DYM* gene and an association between an SNP, rs833497, and schizophrenia was identified (allelic $P=2\times10^{-5}$, in the total sample). *DYM* is the causal gene for Dyggve–Melchior–Clausen syndrome and this study shows the second neuropsychiatric disorder in which the *DYM* gene is involved. The present data support the involvement of Golgi function and vesicular transport in the presynapse in schizophrenia.
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Keywords: association; Dyggve–Melchior–Clausen syndrome; Golgi; postmortem study; SNP

INTRODUCTION

Schizophrenia is a chronic, severe and disabling brain disorder that affects approximately 1% of the world's population. Family and twin studies indicate a strong genetic factor and recent genome-wide association studies provided molecular genetic evidence for a substantial polygenic component to the risk of schizophrenia involving many common alleles of very small effect.[1,2] Some of the genetic factors influencing susceptibility to schizophrenia may also have roles in other phenotypes.

In a recent study,[3] in which mRNA abundance was determined by sequencing mRNA in postmortem cerebellum, gene ontology annotation of genes with significantly altered expression revealed overrepresentation of membrane-associated genes, genes involved in zinc binding or transport, regulation of transcription, Golgi apparatus and vesicle-mediated transport. The authors mentioned that most striking were 23 genes involved in presynaptic vesicular transport/Golgi apparatus or postsynaptic neurotransmission. Meta-analysis of genetic associations revealed nucleotide variants in synaptic vesicular transport/Golgi apparatus genes with schizophrenia (*DTNBP1*, *DISC1*, *DAOA*, *NRG1*).[4] Reelin accumulated in the Golgi and endoplasmic reticulum in some cell bodies of GABAergic neurons in the cortex and hippocampus,[5]and genetic associations of the reelin gene polymorphism and schizophrenia were reported.[6,7]

Recently, dymeclin has been identified to be associated with the Golgi apparatus and with transitional vesicles of the reticulum–Golgi interface and it seems to be involved in cellular vesicle trafficking.[8,9] The *DYM* gene, located in chromosome 18q21.1 and encoding dymeclin, is a causative gene for Dyggve–Melchior–Clausen syndrome, which shows dwarfism and mental retardation. Furthermore, previous studies have identified a putative gene locus for both schizophrenia and bipolar disorder in the 18q21 region.[10,11] The aim of this study was to evaluate genetic associations of polymorphism(s) in the *DYM* gene with schizophrenia.

MATERIALS AND METHODS

All subjects were unrelated and of Japanese descent and were recruited from the main island of Japan. The first sample set was 576 patients with schizophrenia (mean age ± s.d., 51.6 ± 14.8 years; 322 men and 254 women) and 576 control subjects (mean age ± s.d., 46.8 ± 12.5 years; 268 men and 308 women). The second sample set was 1344 patients with schizophrenia (mean age ± s.d.,

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46.7 ± 14.4 years; 733 men and 611 women) and 1344 control subjects (mean age ± s.d., 47.8 ± 13.8 years; 783 men and 561 women). The third sample set was 212 patients with schizophrenia (mean age ± s.d., 37.3 ± 11.4 years; 107 men and 105 women) and 189 control subjects (mean age ± s.d., 37.6 ± 11.5 years; 92 men and 97 women). Consensual diagnosis of schizophrenia was made according to the Diagnostic and Statistical Manual of Mental Disorders, Fourth Edition (American Psychiatric Association, 1994). Control subjects had no history of mental illness and second-degree relatives were free of psychosis in a brief psychiatric interview. This study was approved by the ethics committees of the University of Tsukuba, Niigata University, Fujita Health University, Nagoya University, Okayama University and Seiwa Hospital; and all participants provided written informed consent.

DNA was extracted from blood samples. We genotyped a total of 14 single-nucleotide polymorphisms (SNPs), rs833523, rs357894, rs2044550, rs833497, rs8089472, rs12606288, rs1297381, rs1943000, rs4630621, rs4491603, rs16950465, rs11082743, rs3809924 and rs12606865. The tag SNPs in the gene were selected using the Haploview program (http://www.broad.mit.edu/mpg/haploview/) with the condition of an r^2 threshold of 0.8 and a minor allele frequency of 0.1. SNPs were genotyped by TaqMan genotyping (Applied Biosystems, Foster City, CA, USA). Although the *DYM* gene spans 417 kb, the gene coverage was reached with these 14 SNPs because the gene resided in a large linkage disequilibrium block. Predesigned TaqMan SNP genotyping assays 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 (Eurogentec, Seraing, Belgium), and genotying was performed with an ABI PRISM 7900HT Sequence Detection System (Applied Biosystems).

In this study, we carried out a three-step case–control association procedure; that is, screening and two independent confirmation studies. SNPs with allelic *P*-values for associations of <0.05 were examined in the second case–control sets, and SNPs with allelic *P*-values <0.05 in the second set were examined in the third case–control sets. Significant association was defined when SNPs survived these three-step procedures.

The Hardy–Weinberg equilibrium and an association between SNP and schizophrenia were determined with the Haploview software program (http://www.broad.mit.edu/mpg/haploview/). Genotype-based association was tested with the Cochran–Armitage test for trend.

RESULTS

In the first screening, we genotyped 14 tag SNPs in 1152 individuals (Figure 1). Genotypic distributions of these SNPs are shown in Table 1. Distributions of all SNPs did not differ significantly from the Hardy–Weinberg equilibrium. Nominally significant association was observed in 4 out of 14 SNPs; rs833523 (P=0.005), rs357894 (P=0.004), rs833497 (P=0.007) and rs3809924 (P=0.005).

In the second sample set, these 4 SNPs were genotyped in 2688 individuals. One SNP (rs833497) was significantly associated with schizophrenia (P=0.006, one sided, Table 2). In the third sample set, rs833497 was again genotyped in 404 individuals and the association was confirmed (P=0.006, one-sided, Table 3). In the combined total samples, the allelic *P*-value for association with schizophrenia was 2×10^{-5} (Table 3). The association was observed in both male and female subjects (data not shown).

DISCUSSION

To our knowledge, this is the first report on the association between *DYM* gene variants and schizophrenia. Four SNPs among 14 tag SNPs we examined showed a trend for association in the screening samples (permutation allelic *P*-values from 0.05 to 0.06). Among the four SNPs, an association of SNP 4 (rs833894) with schizophrenia was confirmed in the second and third case–control samples. Thus, the SNP rs833497 was found to be associated with schizophrenia in this study.

The SNP is not likely to exert an important effect on dymeclin function, because rs833497 is located in the last intron of the *DYM* gene, and therefore, it is assumed that the SNP is in linkage disequilibrium with causal SNP(s) for the association. However, rs833497 was in no complete linkage disequilibrium with other SNPs in this study and in the International HapMap database (http://hapmap.ncbi.nlm.nih.gov/). Therefore, we could not predict where the supposed causal variation(s) is. In addition, because no variant in the exons has been identified by the JSNP project (http://snp.ims.u-tokyo.ac.jp/search_Gene.html), we did not perform resequencing of the DNA of our subjects.

The *DYM* gene is located at chromosome 18q21. Previous linkage and cytogenetic studies reported the 18q21 region for both schizophrenia and bipolar disorders.[10,11] However, genome-wide association studies in other populations have not reported a significant association between variants in the *DYM* gene and schizophrenia or bipolar disorders. In the Wellcome Trust Case Control Consortium

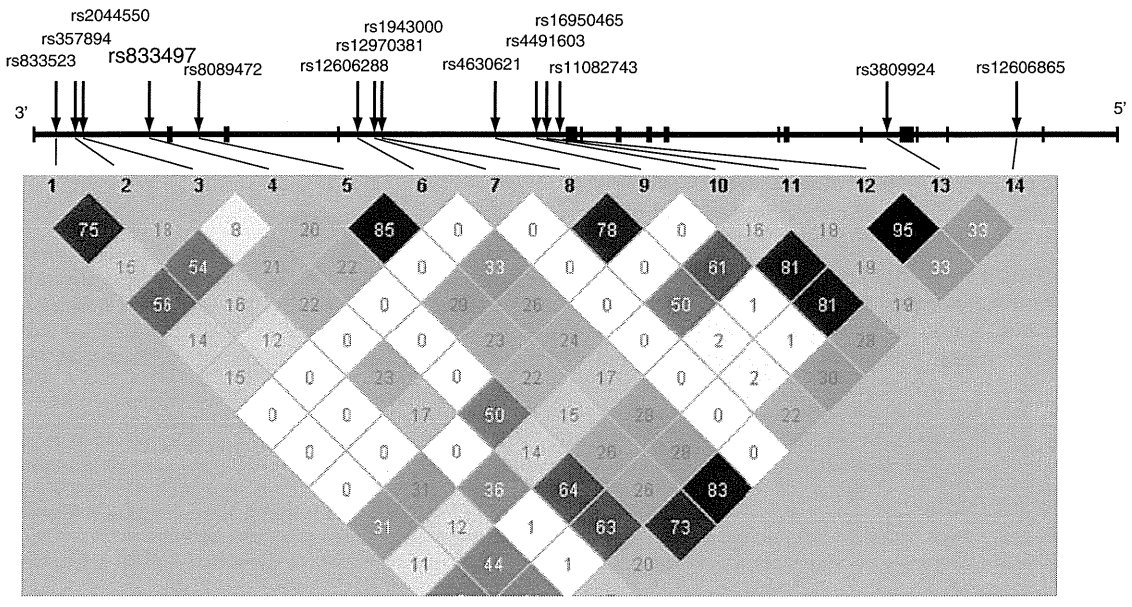

Figure 1 Positions and pairwise linkage disequilibrium (LD) of 14 tag SNPs genotyped in the *DYM* gene.