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

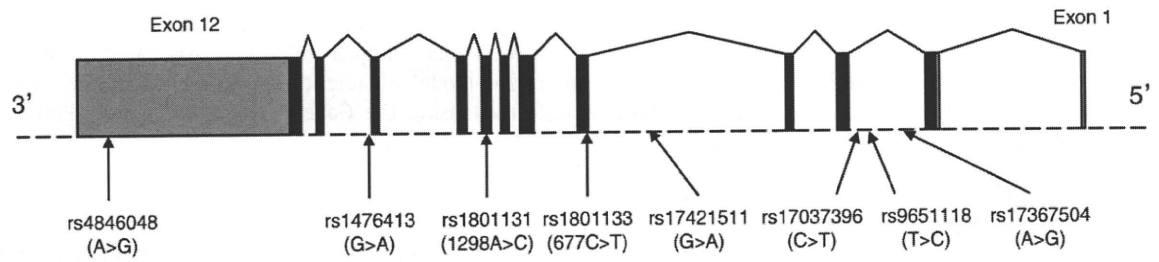

Fig. 1. Genomic structure of *MTHFR*. Black boxes indicate protein-coding regions, while the gray boxes represent the untranslated regions (UTRs). Each box represents *MTHFR* exons. Numbers under the arrows represent the SNP IDs, the tagging SNPs (pairwise tagger: $r^2>0.8$; Haploview 3.32), and the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

Association study of *ubiquitin-specific peptidase 46* (*USP46*) with bipolar disorder and schizophrenia in a Japanese population

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Recently, *ubiquitin-specific peptidase 46* (*Usp46*) has been identified as a quantitative trait gene responsible for immobility in the tail suspension test and forced swimming test in mice. Mice with 3-bp deletion in *Usp46* exhibited loss of 'behavioral despair' under inescapable stresses in addition to abnormalities in circadian behavioral rhythms and the GABAergic system. Considering the face and construct validity as an animal model for bipolar disorder, we explored an association of *USP46* and bipolar disorder in a Japanese population. We also examined an association of *USP46* and schizophrenia. We found nominal evidence for an association of rs12646800 and schizophrenia. This association was not significant after correction for multiple testing. No significant association was detected for bipolar disorder. In conclusion, our data argue against the presence of any strong genetic susceptibility factors for bipolar disorder or schizophrenia in the region *USP46*.
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Keywords: association study; bipolar disorder; schizophrenia; ubiquitin–proteasome system; *USP46*

INTRODUCTION

Biological studies have shown that the ubiquitin–proteasome system, which is highly conserved from yeast to man as the principal means of targeting cytosolic proteins for degradation, has an important role in neuronal function, such as synaptic formation, transmission and plasticity.[1–3] Genetic studies also have implicated the ubiquitin–proteasome system in a range of neuropsychiatric diseases, such as Parkinson's disease,[4] autism spectrum disorders,[5,6] mental retardation,[7–9] bipolar disorder[10,11] and schizophrenia.[11,12]

More recently, quantitative trait locus studies in mice have revealed that *ubiquitin-specific peptidase 46* (*Usp46*) is responsible for negligible immobility in the tail suspension test and forced swimming test, the experimental paradigms for assessing antidepressant activity and depression-like behavior.[13] Usp46 is one of approximately a hundred deubiquitinating enzymes. Protein deubiquitination by deubiquitinating enzymes can either antagonize or facilitate substrate presentation to the proteasome.[2] Deubiquitinating enzymes have also been associated with neurogenetic disorders, including Parkinson's disease, spinocerebellar ataxia. In the aforementioned study,[13] mice with 3-bp deletion in the exon region of *Usp46* exhibit loss of 'behavioral despair' under short-term, inescapable stresses of being suspended by their tail (tail suspension test) or being forced to swim in a water-filled cylinder (forced swimming test). 'Behavioral despair' was a characteristic immobile posture adopted by animals under stresses. Abnormalities in circadian behavioral rhythms and the GABAergic system, both of which are observed in bipolar disorder,[14] were also reported in the mice.[13] Furthermore, the *USP46* locus (4q12) corresponds to the linkage regions for bipolar disorder.[15] Considering the face and construct validity as an animal model for bipolar disorder and the findings of the linkage study, we explored an association of *USP46* with bipolar disorder in a Japanese population.

In addition, recent results from a genome-wide association study[16] and a population-based epidemiological study[17] provided evidence that schizophrenia and bipolar disorder share some common genetic causes. Therefore, we also examined an association between *USP46* and schizophrenia. It should be noted that abnormalities in circadian

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rhythms and the GABAergic system[18] have been reported in schizophrenia as well.

MATERIALS AND METHODS

Subjects

For the bipolar disorder study, 867 cases (mean age, 50.7 ± 14.2 years) and 895 age- and gender-matched controls (49.9 ± 13.5 years) were used. This sample panel was the same as used in the Collaborative Study of Mood Disorder consortium study.[19] Seven laboratories (National Institute of Neuroscience, two laboratories of RIKEN Brain Science Institute, Kohnodai Hospital, Teikyo University, Okayama University and Fujita Health University) provided case and control samples. The proportion of cases with each disorder was 67.5, 31.9 and 0.6% for bipolar I disorder, bipolar II disorder and schizoaffective disorder, respectively. For the schizophrenia study, 715 cases (47.5 ± 14.0 years) and age- and gender-matched 711 controls (46.7 ± 13.1 years) were used. Controls used in the bipolar disorder or schizophrenia studies were independent. All subjects were of Japanese descent. Consensus diagnosis of bipolar disorder or schizophrenia was made according to the criteria of the Diagnostic and Statistical Manual of Mental Disorders, Fourth Edition, by at least two experienced psychiatrists, on the basis of unstructured interviews, available medical records, and information from hospital staff and relatives. Controls were psychiatrically screened using an unstructured interview to exclude subjects with brain disorders or psychotic disorders. This study was approved by the Ethics Committees of all participating institutes. All participants provided written informed consent.

Tagging SNP selection, SNP genotyping and quality control

To test for genetic association, the gene-based approach was implemented. This method implies inclusion of both gene and gene-adjacent regions in the association study.[20] Therefore, the screened region was extended 10 kb upstream of the annotated transcription start site and downstream at the end of the last *USP46* exon. Consulting the HapMap database (release #24, population: Japanese in Tokyo), tagging single-nucleotide polymorphisms (SNPs) were selected to capture common SNPs (minor allele frequency >5%) in the predefined *USP46* locus. Given the linkage disequilibrium structure, seven tagging SNPs were selected, capturing all 30 common SNPs in the *USP46* locus at correlation coefficient (r^2)=1.

Genomic DNA was extracted from leukocytes by using the standard method. SNP genotyping was performed using the TaqMan system (Applied Biosystems, Foster City, CA, USA). PCR was performed using ABI 7900HT Fast Real-time PCR system and fluorescent signals were analyzed using SDS v2.2.1 software (Applied Biosystems).

For quality control, first, deviation from the Hardy–Weinberg equilibrium was checked in controls. Second, we excluded samples with call rates <100% from analyses.

Statistical analyses

The χ^2-test was used to compare the allele or genotype frequencies between cases and controls. Deviation from the Hardy–Weinberg equilibrium was also tested by the χ^2-test. Haplotype frequencies were estimated in a two- and three-marker sliding window manner by expectation maximization algorithm. Log likelihood ratio tests were performed for global *P*-values with COCAPHASE program in the UNPHASED v2.403 program (http://www.mrc-bsu.cam.ac.uk/personal/frank/software/unphased/).[21] All *P*-values reported were two-tailed. Statistical significance was defined as $P<0.05$. Power calculation was conducted with CaTS software (http://www.sph.umich.edu/csg/abecasis/CaTS/download.html).

RESULTS

All tagging SNPs were in Hardy–Weinberg equilibrium in controls. After excluding samples with call rates <100%, 845 cases and 869 controls in the bipolar disorder study, and 699 cases and 701 controls in the schizophrenia study remained for subsequent analyses. Assuming a multiplicative genetic model and a disease prevalence of 1%, power calculations showed that our sample had sufficient power (>80%) to detect gene-wide significant associations with genotype-relative risk values of 1.21–1.58 (minor allele frequency, 0.036–0.496) and 1.23–1.58 (minor allele frequency, 0.044–0.486) in bipolar disorder and schizophrenia, respectively. The linkage disequilibrium structures around *USP46* locus in 1570 control samples (869 controls in the bipolar disorder study+701 controls in the schizophrenia study) are shown in Table 1 and are highly similar to those of the JPT HapMap samples, ensuring that our genotyping were conducted correctly. The results of analyses are shown in Tables 2 and 3. We found nominal evidence for an association of rs12646800 with schizophrenia (allelic $P=0.04$, genotypic $P=0.01$). However, this association was not significant after Bonferroni correction. In bipolar disorder, no significant association was detected in allele-/genotype-/haplotype-wise analyses.

DISCUSSION

Although we could not detect evidence of a strong association of the *USP46* locus with bipolar disorder or schizophrenia in a Japanese population, these results could be interpreted in several ways. First, the results could indicate that there is no relevance of the *USP46* locus to these psychiatric disorders. Second, although this study was based on the common disease–common variant model, the genetic architecture of psychiatric disorders might be closer to the multiple rare variant model, making detection of causal variants difficult. Concerning this, on the basis of the epidemiological data and evolution theory, Uher[22] recently argues that severe mental illnesses, including schizophrenia that confer strong reproductive disadvantage, are likely to have a large and pleiotropic contribution from rare variants of recent origin. Third, it is possible that we overestimated the effect size of disease-related variants; that is, this study might be underpowered to detect variants

Table 1 Linkage disequilibrium analysis of *USP46*

SNP	*rs346005*	*rs10034164*	*rs2244291*	*rs12646800*	*rs6554557*	*rs17675844*	*rs10517263*
rs346005		1.00	1.00	1.00	1.00	0.99	1.00
rs10034164	0.14		1.00	1.00	0.99	1.00	1.00
rs2244291	0.43	0.06		1.00	0.98	0.99	1.00
rs12646800	0.04	0.01	0.02		1.00	1.00	1.00
rs6554557	0.14	0.97	0.06	0.01		1.00	1.00
rs17675844	0.10	0.02	0.23	0.00	0.02		1.00
rs10517263	0.09	0.62	0.04	0.00	0.61	0.01	

Abbreviations: SNP, single-nucleotide polymorphism; *USP46*, *ubiquitin-specific peptidase 46*.
Values shown above the diagonal are D′ and values shown below are r^2. Data of 1570 controls (control in bipolar disorder analysis, *N*=869; controls in schizophrenia analysis, *N*=701) were used for the calculation.

Table 2 Allele-/genotype-/haplotype-wise analyses in bipolar disorder

		Genotype counts						Single SNP		Haplotype-wise[a]	
		Case (N=845)			Control (N=869)						
dbSNP	*M/m*	*M/M*	*M/m*	*m/m*	*M/M*	*M/m*	*m/m*	*Allele -wise*	*Genotype -wise*	*2-window*	*3-window*
rs346005	A/C	213	427	205	215	432	222	0.61	0.83		
										0.26	
rs10034164	A/G	632	197	16	674	179	16	0.22	0.39		0.30
										0.34	
rs2244291	A/G	419	363	63	413	378	78	0.25	0.45		0.34
										0.35	
rs12646800	G/A	769	72	4	807	61	1	0.10	0.19		0.42
										0.25	
rs6554557	T/G	630	199	16	669	183	17	0.30	0.46		0.30
										0.37	
rs17675844	T/G	713	128	4	716	147	6	0.25	0.50		1.00
										1.00	
rs10517263	G/C	709	130	6	739	122	8	0.62	0.66		

Abbreviations: M, major allele; m, minor allele; SNP, single-nucleotide polymorphism.
[a]Sliding window analysis, rare haplotype threshold 10%.

Table 3 Allele-/genotype-/haplotype-wise analyses in schizophrenia

		Genotype counts						Single SNP		Haplotype-wise[a]	
		Case (N=699)			Control (N=701)						
dbSNP	*M/m*	*M/M*	*M/m*	*m/m*	*M/M*	*M/m*	*m/m*	*Allele -wise*	*Genotype -wise*	*2-window*	*3-window*
rs346005	A/C	169	342	188	170	342	189	1.00	1.00		
										0.95	
rs10034164	A/G	526	160	13	533	155	13	0.76	0.94		0.93
										0.92	
rs2244291	A/G	346	278	75	336	293	72	0.75	0.74		0.84
										0.55	
rs12646800	G/A	661	36	2	640	61	0	0.04	0.01		0.82
										0.79	
rs6554557	T/G	527	157	15	533	156	12	0.67	0.83		0.89
										0.75	
rs17675844	T/G	579	115	5	571	122	8	0.41	0.62		1.00
										1.00	
rs10517263	G/C	590	101	8	590	106	5	0.93	0.67		

Abbreviations: M, major allele; m, minor allele; SNP, single-nucleotide polymorphism.
[a]Sliding window analysis, rare haplotype threshold 10%.

with small effect. For example, the range of odds ratios was 1.15–1.24 in seven markers, which were recently reported to show genome-wide significant association with schizophrenia.[23] Although the association between rs12646800 and schizophrenia was not significant after Bonferroni correction in our study, this correction may be too stringent because of the presence of linkage disequilibrium. For this reason, we checked whether there was an association of *USP46* with schizophrenia in a recent genome-wide association study. In the genome-wide association study by Need *et al.*[24], *USP46* locus included one SNP nominally associated with schizophrenia in a Caucasian population (rs2244291; allelic P=0.027). Although rs2244291 is not associated with schizophrenia in our study (allelic P=0.75, genotypic P=0.74) and rs12646800 is not polymorphic in HapMap Caucasian samples, it should be noted that rs2244291 is only ~200 bp away from rs12646800. This might point to the relevance of this region within *USP46* to risk for schizophrenia.

In addition, we searched two databases for further evidence of association of *USP46* with bipolar disorder or schizophrenia. First, we referred to the Stanley Medical Research Institute Online Genomics Database (https://www.stanleygenomics.org/) to examine the differences in USP46 expression in post-mortem brains.[25] Although we did not find a significant difference in USP46 expression between patients with schizophrenia and controls in a combined analysis of the results from 16 studies, we detected evidence of a trend for association in USP46 expression change between patients with bipolar disorder and controls in a combined analysis of the results from 18 studies (P=0.089), with USP46 expression in bipolar disorder reduced. Second, we referred to the Database of Genomic Variants[26] in search

of copy number variations with functional implication at *USP46* locus. Although we could not find copy number variations in this locus, it cannot be ruled out that unknown copy number variations located in this locus have an important role in the etiology of psychiatric disorders.

In conclusion, our data argue against the presence of any strong genetic susceptibility factors for bipolar disorder or schizophrenia in the region *USP46*. However, considering the limitations of this genetic association study and supportive evidence from various datasets, expansion of samples or resequencing strategy would be required for a more conclusive result.

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Identification of Novel Candidate Genes for Treatment Response to Risperidone and Susceptibility for Schizophrenia: Integrated Analysis Among Pharmacogenomics, Mouse Expression, and Genetic Case-Control Association Approaches

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Background: Pharmacogenomic approaches based on genomewide sets of single nucleotide polymorphisms (SNPs) are now feasible and offer the potential to uncover variants that influence drug response.

Methods: To detect potential predictor gene variants for risperidone response in schizophrenic subjects, we performed a convergent analysis based on 1) a genomewide (100K SNP) SNP pharmacogenetic study of risperidone response and 2) a global transcriptome study of genes with mRNA levels influenced by risperidone exposure in mouse prefrontal cortex.

Results: Fourteen genes were highlighted as of potential relevance to risperidone activity in both studies: *ATP2B2, HS3ST2, UNC5C, BAG3, PDE7B, PAICS, PTGFRN, NR3C2, ZBTB20, ST6GAL2, PIP5K1B, EPHA6, KCNH5,* and *AJAP1*. The SNPs related to these genes that were associated in the pharmacogenetic study were further assessed for evidence for association with schizophrenia in up to three case-control series comprising 1564 cases and 3862 controls in total (Japanese [JPN] 1st and 2nd samples and UK sample). Of 14 SNPs tested, one (rs9389370) in *PDE7B* showed significant evidence for association with schizophrenia in a discovery sample (p_{allele} = .026 in JPN_1st, two-tailed). This finding replicated in a joint analysis of two independent case-control samples (p_{JPN_2nd+UK} = .008, one-tailed, uncorrected) and in all combined data sets (p_{all} = .0014, two-tailed, uncorrected and p_{all} = .018, two-tailed, Bonferroni correction).

Conclusions: We identified novel candidate genes for treatment response to risperidone and provide evidence that one of these additionally may confer susceptibility to schizophrenia. Specifically, *PDE7B* is an attractive candidate gene, although evidence from integrated methodology, including pharmacogenomics, pharmacotranscriptomic, and case-control association approaches.

Key Words: Expression: PDE7B, pharmacogenomics, risperidone, schizophrenia

Schizophrenia is a severe psychiatric disorder with a lifetime risk of approximately 1%. With its early onset, typically in late teens to early 20s, frequent relapse and chronic course, schizophrenia imposes a considerable burden on sufferers, their families, and society. Worldwide, it is a major source of morbidity, but it is often overlooked that it is also associated with a considerable truncation in life span, the mortality rate in individuals with schizophrenia being more than twice that of the age- and sex-matched population (1). A large number of antipsychotics have been developed as treatment agents. However, individual response to these drugs is highly variable, and identifying the optimal treatment for any patient is often a trial and error process that can span many years and even then, response is often poor. There is a pressing need both to identify new treatments and to attempt to improve the information based on which response to treatment can be predicted.

Genetic factors are generally assumed to contribute to variable treatment response (2), and on this basis, a number of pharmacogenetic studies have been performed. Here, the aim was to detect DNA sequence predictors for treatment response. Most studies have focused on genes encoding neurotransmitter receptors, such as dopamine or serotonin receptors, the logic being that antipsychotics usually have high affinities with members of these classes of receptor. Although a number of variants have been correlated with treatment response in several stud-

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ies—for example, dopamine D2 and D3 receptor variants (2)—there are no definitive predictors of response.

Pharmacogenetics has been driven by a candidate gene approach. This approach has the disadvantage that targets for study are limited by our current understanding of the mechanisms of drugs, and therefore, this method cannot identify unsuspected predictor genes. Approaches that are independent of prior functional hypotheses of gene action based on genome-wide surveys of SNPs are, however, now feasible. The genome-wide approach has its disadvantages, but one of the most important is that, with effectively random sets of SNPs, the low prior probability that any is truly associated with disease requires a stringent type I error rate to control the enormous potential for reporting false positives. One way to address this issue is to use very large (and therefore highly powered) studies in which such stringent statistical support might realistically be achieved. Another approach that is more economical in genotyping costs is to undertake multistage analysis in which candidate variants from a screening sample are validated by replication in other data sets (3). However, because the samples for pharmacogenomics require a large amount of clinical data and are preferably prospective, large samples are difficult to collect.

Another approach is to try to enhance the prior probability for a given gene by integrating pharmacogenomic data with other sources of data—for example, from studies of gene expression (4). Under the hypothesis that genes related to drug response may be regulated by exposure to that drug, genes in which expression is altered in animals exposed to that drug have a higher probability of being genuinely associated in a pharmacogenetic study than any random gene. If this is correct, genes in which expression is altered by drug exposure require less stringent statistical support.

We aimed to detect predictor genes for risperidone response in schizophrenic patients using this convergent approach (4). Specifically, we compared data from a pharmacogenetic study based on first-episode, previously drug-naive subjects with schizophrenia who were treated with risperidone with data from a pharmacotranscriptomic study based on mice exposed to the same drug. Moreover, candidate variants from genes implicated by convergent data were also tested for evidence for association to schizophrenia per se because variants that are related to drug response may also be related to disease risk (5). Evidence that this occurs can be considered an additional independent line of circumstantial support that the convergence between the pharmacogenetic and transcriptomics does not merely reflect chance.

Methods and Materials

Subjects and Collection of Clinical Data

We performed an open-labeled pharmacogenetic study involving 108 first-episode, previously antipsychotic-naive schizophrenic patients. All received risperidone monotherapy after enrollment. Details are described elsewhere (6,7). Briefly, patients were entered into the study if they 1) met DSM-IV-TR criteria for schizophrenia (and then remained in follow-up to at least 6 months), 2) were physically healthy and had all laboratory parameters within normal limits, and 3) had neither a current nor a past DSM-IV-TR diagnosis of mood disorders or substance abuse. Consensus diagnoses were made by at least two experienced psychiatrists on the basis of unstructured interviews with patients and families and review of medical records. Duration of untreated psychosis (DUP) was defined as the period from the onset of psychotic symptoms to that of first antipsychotic exposure. Sixty subjects were recruited from outpatient clinics, and 48 subjects were treated as inpatients.

Subjects received risperidone monotherapy (starting dosage: .5–4 mg/day, mean starting dosage: 2.5 mg), and dosage was adjusted in accordance with symptomatic response by trained psychiatrists (1–8 mg/day, mean dosage: 3.4 mg at 8 weeks) for 8 weeks. Patients with insomnia were prescribed brotizolam, .25 mg or .5 mg, at bedtime. No other psychotropic drugs were permitted.

Clinical symptoms were evaluated at the first visit and after 8 weeks of treatment by the use of the Positive and Negative Syndrome Scale (PANSS). Evaluations were carried out by qualified psychiatrists and psychologists (the interrater reliability was measured by intraclass correlation coefficient was .90, unpublished data).

The clinical characteristics of subjects that we used as potential covariates were selected from another report (8): sex (57 male, 51 female), age (mean 30.2 ± 9.5 years), DUP (1.5–32 months, mean 7.6 ± 7.1 months), and baseline PANSS total score (mean 83.0 ± 22.9).

Samples used in the schizophrenia case-control association analysis consisted of three sets: (1) JPN_1st: this was used for identifying genes of potential interest and comprised 540 patients with schizophrenia (275 male and 265 female; aged 43.3 ± 15.0 years) and 425 healthy controls (236 male and 189 female; aged 36.3 ± 13.9 years) from the Japanese population; 2) JPN_2nd sample (used to follow up genes of interest) comprised 545 patients with schizophrenia (282 male and 263 female; aged 50.7 ± 14.9 years) and 500 controls (279 male and 221 female; aged 40.8 ± 15.4 years) from the Japanese population; 3) Additional follow-up data for SNPs of interest were extracted from a UK genomewide association study (GWAS) of schizophrenia comprising 479 patients with schizophrenia and 2937 controls from the UK population (9).

Controls in the Japanese population were screened for past history of mental disorders. All individuals were unrelated. After explanation of the study, written informed consent was obtained from each subject. This study was approved by the Ethics Committee at Fujita Health University, University of Occupational and Environmental Health, Nagoya University Graduate School of Medicine, Osaka University Graduate School of Medicine and by multiple ethics committees across the UK where sample recruitment was performed.

Microarray Experiments

See also Methods in Supplement 1.

SNP Chip. Genomewide genotyping was carried out using Illumina Sentrix human 1 Genotyping BeadChip (109,363 SNPs randomly distributed throughout the genome) according to the manufacturer's instructions (Illumina, San Diego, California). Details are given in the Supplement 1.

Mouse Expression Chip. We compared mRNA levels of the prefrontal cortex (PFC) between control ($n = 3$) and risperidone-exposed mice (2.4 mg/kg given orally, once a day for 21 days, $n = 3$). Affymetrix Mouse Gene 1.0 St. Array, which profiles the expression of 28,853 genes (Affymetrix, Santa Clara, California), was used to measure the amount of mRNA.

The procedures involving animals and their care were conducted in conformity with the international guidelines, Principles of Laboratory Animal Care (National Institutes of Health Publication 85-23, revised 1985).

Experimental Procedures and Statistical Analysis

Study 1: Pharmacogenomics. Quality control (QC) regarding population stratification (Figure S1 in the Supplement 1),

Hardy-Weinberg equilibrium (HWE), genotyping rate, and minor allele frequency was conducted by PLINK (10). Details are described in Supplement 1.

After QC, 99 samples (51 males and 48 females) and 62,935 autosomal SNPs (a mean call rate of 99.2%, indicating a high rate of successful genotyping) were analyzed to evaluate the effect of each SNP on antipsychotic response to risperidone.

To evaluate the effect of each SNP on antipsychotic response to risperidone, multiple regression analysis was carried out with a dependent variable [% PANSS change = 100 × ((PANSS at week 0) − (PANSS at Week 8))/PANSS at Week 0] and independent variables that included sex, age, duration of illness, initial PANSS score, and the genotype of each polymorphism. Each genotype was assessed using dominant, recessive, and multiplicative genetic models, respectively.

To calculate the best empiric p values based on the most significant result in each genetic model, we generated 1 million simulated data sets by randomizing the PANSS changes (the covariates stay with the genotypes) with respect to the GWAS data. This approach retains the linkage disequilibrium (LD) relationships between SNPs, and therefore allows for the appropriate degree of nonindependence in the data sets. The same multiple regression analysis model as applied to the real data were applied to each SNP in each permuted data set, and the empiric significance for a SNP was the proportion of the simulated data sets in which the test statistic was equal to, or greater than, that observed in the true data set (11–15).

SNPs were annotated to the closest genes with an up- and downstream span of 20 kb by WGAViewer (16).

Study 2: Mouse Expression Assay. In the mouse expression assay, data sets passing QC were normalized using GeneChip Operating Software (Affymetrix) and the raw intensity values exported for further analysis. Only genes called present based on Affymetrix detection p value for the presence of each gene on either chip were included. A t test was performed to assess the statistical significance of genes in which expression differed between control and risperidone-exposed mice. Power analysis was carried out using PowerAtlas (17). Our data set provides expected discovery rate (corresponding to power) of .37, an expected proportion of true positives (PTPs) of .72, and an expected proportion of true negatives of .80 at alpha set at .05. A major aim of this study was to prioritize genes showing convergent evidence in the pharmacogenomic study, thus we consider a high PTP optimal.

These data were submitted to CIBEX (http://cibex.nig.ac.jp/index.jsp, accession number: CBX77).

Study 3: Checking Overlap Results Between Pharmacogenomics (Study 1) and Mouse Expression Assay (Study 2). We checked candidate SNPs from the genes that showed convergent evidence for relevance to risperidone action from Study 1 and Study 2. Candidate genes were defined as follows: 1) genes for which there was at least one SNP with p values less than 5.0×10^{-4} in the pharmacogenomic study and in which expression significantly differed between groups at $\alpha < .05$ and 2) genes with much stronger evidence for $p < 1.0 \times 10^{-4}$ but that had weaker evidence for association in the pharmacogenomic study ($p < .05$).

Study 4: Case-Control Association Analysis of Strong Candidate Genes from Pharmacogenomics and Mouse Expression Assay. The candidate SNPs from Study 3 were further assessed for evidence for association with schizophrenia. These SNPs were genotyped by TaqMan assay (Applied Biosystems, California) in the Japanese case-control samples. Genotypes for the SNP in *PDE7B* in the UK samples were extracted from the Affymetrix GWAS data (9) after confirmation of good-quality cluster plots.

Genotype deviation from HWE was evaluated by a goodness of fit chi-square test. Marker-trait association was evaluated for allele/genotype-wise using standard contingency tables (SPSS 15.0, SPSS, Tokyo, Japan).

For SNPs analyzed in multiple samples, we conducted a meta-analysis using a random-effects model. Heterogeneity was measured using a Q statistic test in the combined studies. Odds ratios (ORs) were pooled using DerSimonian and Laird methods. The significance of the pooled OR was determined using a Z test. All data were analyzed using an R package, meta (http://www.r-project.org/index.html).

Results

Possible Predictor SNPs for Risperidone Treatment: From Pharmacogenomic Result (Study 1)

Among the 62,935 SNPs we examined in the pharmacogenomics study, 51,550 SNPs were annotated to 14,655 genes (annotation span: 5′ or 3′ ± 20 kb). For a number of genes, we had multiple SNPs with p values less than 5.0×10^{-4} because of the high LD among genotyped markers. Where this occurred, we list only the strongest associated SNP from that gene (the top 10 hits and SNPs with p value less than 5.0×10^{-4} in Table 1 and Table S1 in Supplement 1, respectively).

Table 1. Predictor Genes in the Pharmacogenomics (Top 10)

Ranking	SNP	Chr	Position[a]	Closest Gene[b]	p Value (Pharmacogenomics)
1	rs2289273	3	10,388,601	*ATP2B2*	1.60×10^{-5}
2	rs234091	1	183,186,172	*FAM129A*	2.00×10^{-5}
3	rs241202	8	28,689,604	*INTS9*	3.20×10^{-5}
4	rs4340422	19	48,604,802	*TEX101*	5.00×10^{-5}
5	rs6682786	1	23,615,883	*TCEA3*	7.30×10^{-5}
6	rs1001220	7	72,748,539	*WBSCR22*	7.70×10^{-5}
7	rs3829241	11	68,611,939	*TPCN2*	8.90×10^{-5}
8	rs460473	16	22,740,528	*HS3ST2*	1.03×10^{-4}
9	rs9792264	8	135,640,117	*ZFAT*	1.10×10^{-4}
10	rs6443999	3	186,056,249	*VPS8*	1.17×10^{-4}

Chr, chromosome; SNP, single nucleotide polymorphism.
[a]Based on Ensemble *Homo sapiens* Version 54.36p (NCBI36).
[b]SNPs are annotated to the closest genes with ± 20-kb span.

Table 2. Overlap Genes Based on the Pharmacogenomcis ($p < 5.0 \times 10^{-4}$) with Mouse Expression Assay ($p < .05$)

Ranking	SNP	Chr	Position[a]	Closest Gene[b]	*p* Value (Pharmacogenomics)	*p* Value[c] (Mouse Expression)	Fold Change
1	rs2289273	3	10,388,601	*ATP2B2*	1.60×10^{-5}	.000710	.504
8	rs460473	16	22,740,528	*HS3ST2*	1.03×10^{-4}	.00600	.259
28	rs3775003	4	96,390,234	*UNC5C*	2.20×10^{-4}	.0132	1.85
32	rs196290	10	121,398,061	*BAG3*	2.81×10^{-4}	.0283	1.33
35	rs9389370	6	136,472,958	*PDE7B*	2.88×10^{-4}	.00806	.710
53	rs1356787	4	57,012,104	*PAICS*	4.26×10^{-4}	.0368	.660
54	rs4641299	1	117,284,884	*PTGFRN*	4.27×10^{-4}	.00160	.283

Chr, chromosome; SNP, single nucleotide polymorphism.
[a]Based on Ensemble *Homo sapiens* Version 54.36p (NCBI36).
[b]SNPs are annotated to the closest genes with ± 20-kb span.
[c]Comparison between risperidone-treated mice ($n = 3$) and saline-treated mice ($n = 3$).

We also looked specifically in our data for support for genes recently suggested as associated with iloperidone based on the only other available antipsychotic GWAS data set (18) and candidate genes implicated in earlier studies (2) including *DRD2*, *DRD3*, *HTR2A*, and others (Tables S2 and S3 in Supplement 1). No strong evidence for association to any of these was found in our pharmacogenomics data set.

Genes Influenced by Risperidone Exposure in Mouse PFC (Study 2)

We examined 22,556 probes in 12,706 genes in RNA extracted from the PFC of mice treated with either risperidone or with vehicle. Of these, 754 (5.9%) and 2227 (17.5%) genes had at least one probe that showed nominally significant differences at $p <$ .01 and .05, respectively, a rate much higher than chance. The top genes with *p* value less than 5.0×10^{-4} are presented in Table S4 in Supplement 1.

Overlapping Genes Between Pharmacogenomic and Mouse Expression Assays (Study 3)

We looked to see whether the pharmacogenetic data (excluding 14 SNPs that could not be annotated to the closest gene) and expression overlapped. Seven genes containing nominally significant alteration in expression in mice also contained SNPs with *p* value less than 5×10^{-4} (Table 2). The relation between PANSS changes and physical locations of each SNP and the genotype effects to risperidone response can be seen in Figures S2 and S3 and Table S5 in Supplement 1. In addition, we found seven genes that met the more stringent threshold for expression change in the mouse and that had at least one significant SNP ($p < .05$) in the pharmacogenomic data (Table 3). It should be stressed these SNPs were not strongly associated with treatment response ($p = .0047$-$.0472$).

Consequently 14 SNPs were further assessed for case-control association analysis in Study 4.

Examining Candidate SNPs as Susceptibility Factor for Schizophrenia (Study 4)

The 14 candidate SNPs in genes showing convergent evidence from Study 3 were further tested for association with schizophrenia (Table 4). For rs242056, a proxy for rs2071999 in *AJAP1*, the genotypes significantly deviated from HWE in controls ($p = .0016$). This SNP was therefore excluded.

Of the remaining 13 SNPs, a single SNP (rs9389370) in *PDE7B* showed a nominally significant association in the JPN_1st case-control sample ($p_{\text{allele}} = .026$, two-tailed). In an attempt to extend this putative association, we used two other samples. In the second Japanese sample, we obtained significant evidence for association (second set, $P_{\text{allele}} = .02$, one-tailed) and a nonsignificant trend in the UK sample ($p_{\text{allele}} = .07$, one-tailed) (Table 4). Meta-analysis of the two replication data sets showed significant evidence for association ($p_{\text{JPN2nd+UK}} = .008$, one-tailed). As expected, in all data sets combined, the evidence was stronger than observed in the screening sample alone ($p_{\text{all}} = .0014$, two-tailed, uncorrected; $p = .018$, 13 times Bonferroni correction for number of SNPs tested in Study 4) with no evidence for heterogeneity ($p = .56$; Table 5).

Discussion

Combined Analysis as a Tool for Prioritizing Candidate Genes for Pharmacogenomics and Susceptibility

Genomewide approaches to pharmacogenomics have the capacity to provide novel insights into mechanisms and predictors of drug response. However, a major concern of this approach, which is not specific to pharmacogenomics, relates to balancing the need to set a stringent threshold for the type I error rate against the desire to achieve power to detect findings at that threshold. Unless the genetic effect sizes in pharmacogenetics are substantially greater than is typical for complex diseases (19), the sorts of sample sizes currently available for studies of

Table 3. Overlap Genes Based on the Mouse Expression Assay ($p < 1.0 \times 10^{-4}$) with the Pharmacogenomics ($p < .05$)

Ranking	Gene	Probe ID	Fold Change	*p* Value (Mouse Expression)	*p* Value (Pharmacogenomics)	SNP ID
2	*Nr3c2*	1435991-at	5.86	2.23×10^{-6}	.0297	rs2070951
3	*Zbtb20*	1439278-at	4.94	5.04×10^{-6}	.0230	rs9883949
4	*St6gal2*	1434819-at	.23	6.52×10^{-6}	.0102	rs1448110
7	*Pip5k1b*	1450389-s-at	2.46	1.02×10^{-5}	.0472	rs1414944
8	*Epha6*	1421527-at	3.18	1.46×10^{-5}	.0047	rs727229
9	*Kcnh5*	1441742-at	.44	2.72×10^{-5}	.0305	rs10141458
24	*Ajap1*	1438662-at	.66	9.21×10^{-5}	.0208	rs2071999

SNP, single nucleotide polymorphism.

Table 4. Case-Control Analysis of the Candidate SNPs from the Pharmacogenomics and Mouse Expression Data

SNP	Sample	Proxy SNPs	Phenotype	N	Genotype M/M	Genotype M/m	Genotype m/m	*p* Value Allele	*p* Value Genotype	*p* Value HWE	MAF
ATP2B2	JPN_1st		Case	536	275	208	53	.676	.184	.14	29.3
rs2289273			Control	417	209	179	29			.26	28.4
HS3ST2	JPN_1st		Case	538	163	263	112	.408	.682	.76	45.3
rs460473			Control	407	117	196	94			.50	47.2
UNC5C	JPN_1st		Case	540	336	178	26	.249	.136	.70	21.3
rs3775003			Control	406	231	159	16			.07	23.5
BAG3	JPN_1st		Case	539	234	243	62	.609	.877	.93	34.0
rs196290			Control	407	183	180	44			.98	32.9
PDE7B	JPN_1st		Case	535	259	222	54	**.0255**	.0738	.53	30.8
rs9389370			Control	422	229	165	28			.81	26.2
	JPN_2nd		Case	536	278	200	58	**.0214**[a]	.0966	.018	29.5
			Control	500	281	183	36			.41	25.5
	UK		Case	478	181	226	71	.0672[a]	.327	.97	38.5
			Control	2,932	1203	1348	381			.91	36.0
PAICS	JPN_1st		Case	540	181	274	85	.662	.808	.27	41.1
rs1356787			Control	424	134	223	67			.10	42.1
PTGFRN	JPN_1st		Case	535	319	180	36	.222	.219	.13	23.6
rs4641299			Control	413	255	141	17			.65	21.2
NR3C2	JPN_1st		Case	534	295	206	33	.284	.509	.71	25.5
rs2070951			Control	414	213	173	28			.37	27.7
ZBTB20	JPN_1st		Case	537	234	245	58	.494	.420	.61	33.6
rs9883949			Control	423	169	211	43			.05	35.1
ST6GAL2	JPN_1st	rs2241991	Case	533	224	246	63	.936	.814	.72	34.9
rs1448110		$r2 = 1$	Control	409	169	196	44			.25	34.7
PIP5K1B	JPN_1st		Case	536	173	275	88	.550	.764	.22	42.1
rs1414944			Control	420	145	208	67			.6	40.7
EPHA6	JPN_1st		Case	539	146	274	119	.877	.949	.65	47.5
rs727229			Control	419	110	217	92			.44	47.9
KCNH5	JPN_1st		Case	539	169	265	105	.224	.476	.95	44.1
rs10141458			Control	413	142	201	70			.94	41.3
AJAP1	JPN_1st	rs242056	Case	—	—	—	—	—	—	—	—
rs2071999		$r2 = .46$	Control	418	191	161	66			.0016	35.0

Bold numbers represent significant *p* value.

JPN_1st, first Japanese sample; JPN_2nd, second Japanese sample; MAF, minor allele frequency; HWE, Hardy-Weinberg equilibrium; M, major allele, m, minor allele; SNP, single nucleotide polymorphism; UK, United Kingdom sample.

[a]Based on one-tailed analysis.

antipsychotics have no realistic prospect of attaining the sorts of levels of significance suggested for genomewide significance (7.2×10^{-8} or 1×10^{-7}) (20,21). Although it is at least possible that the typical effects on gene expression of drugs may be much more substantial than that of SNPs on disease risk, broadly similar balances of power and type I error also apply to our genomewide expression study. Therefore, with the aim of prioritizing our findings, we attempted to cross-validate the top findings from our study using independent approaches as suggested (4). Our methods of prioritizing our findings were based on two hypotheses; one that the most highly significant sets of SNPs from our pharmacogenetic study of risperidone are likely to be enriched among genes whose expression is altered by that drug (and vice versa), the other that SNPs related to drug response may also be enriched among SNPs associated with disease. To what extent these hypotheses are correct is currently unknown.

From our data, we found 14 markers in genes that showed some degree of overlapping support in the pharmacogenomics

Table 5. Meta-Analysis of rs9389370 in PDE7B

Analysis	Sample	OR	95% CIs Lower Limit	95% CIs Upper Limit	*p* Value
	JPN_1st	1.26	1.03	1.54	**.0255**
	JPN_2nd	1.22	1.01	1.48	**.0214**[a]
	UK	1.11	.967	1.28	.0672[a]
Meta (Replication)	JPN_2nd+UK	1.15	1.03	1.29	**.0082**[a]
Meta (All)	JPN1st+JPN2nd+UK	1.17	1.06	1.30	**.0014**

Bold number represent significant *p* value.

CI, confidence interval; JPN_1st, first Japanese sample; JPN_2nd, second Japanese sample; OR, odds ratio; UK, United Kingdom Sample.

[a]Based on one-tailed analysis.

and mouse expression experiments. These top convergent candidate genes have no previous support for association with schizophrenia or risperidone response and are thus novel candidates for antipsychotic response. However, at present, they have no clinical utility in terms of predicting treatment response, and independent replication using other samples will be required. Moreover, even if replicated, the potential clinical utility for pharmacogenetics is questionable because the effect sizes in each case are small, although it is conceivable given the limited coverage of each gene that the true functional variants have much stronger effects.

Another method for prioritizing genes from genomewide data are to apply a gene ontology (GO) based approach to investigate whether sets of findings tend to converge on particular biological pathways or functions. Our previous experience of GO category analysis suggests that with respect to genetic data, these require large data sets (22). Nevertheless, in response to an anonymous reviewer's comments, for interested readers, we provide the results of our GO category analyses based on ALIGATOR (22) and David Bioinformatics Resources 2008 (http://david.abcc.ncifcrf.gov/) in Supplement 1. Although a number of categories were observed to be significant in each analysis (Tables S6 and S7 in Supplement 1), there is no overlap between the results of the two analytic approaches. Moreover, our favored approach based on ALIGATOR did not reveal any categories that were significant after correction for multiple testing, so it is likely that all of those findings represent chance positives.

Possible Predictor SNPs for Response to Risperidone

In this study, several genes were detected as possible novel predictors for treatment response to risperidone: *ATP2B2*, *HS3ST2*, *UNC5C*, *BAG3*, *PDE7B*, *PAICS*, *PTGFRN*, *NR3C2*, *ZBTB20*, *ST6GAL2*, *PIP5K1B*, *EPHA6*, *KCNH5*, and *AJAP1*. Because the multiple testing burden in SNPs is more severe, our primary analysis included selecting genes based on the more stringent thresholds in the pharmacogenomics data (Table 2) and were additionally shown to have altered expression in the mouse expression study. However, in response to review, we additionally provide data for much more weakly associated SNPs that have highly significant expression changes in the mouse brain (Table 3). Given the weak evidence for most of the latter group of SNPs, we think those are most likely to be chance positives but report the findings for others to test.

Among genes of particular interest in Table 2 is *ATP2B2*, which encodes one of four isoforms of the plasma membrane Ca^{2+} pumps of mammalian cells, showed both the strongest statistical association with treatment response ($p = 1.60 \times 10^{-5}$) and was among those genes that had the strongest association with differential expression because of exposure to risperidone ($p = .00071$). The product of this gene is thought to be involved in neurodevelopment (23) because of its influence on Ca^{2+} homeostasis and Ca^{2+} signaling. This in turn regulates multiple neuronal functions, including synaptic transmission, plasticity, and cell survival (24). Interestingly, several of the other genes with convergent evidence for a role in risperidone response might also be related to neurodevelopment via association with netrin (*UNC5C*) (25,26), interaction with heat shock proteins (*BAG3*) (27,28), cyclic adenosine monophosphate (cAMP) systems (*PDE7B*; details discussed later), glucocorticoids (*NR3C2*) (29), and ephirin (*EPHA6*) (30). Given the neurodevelopmental hypothesis of schizophrenia (31) and evidence that second-generation antipsychotics, including risperidone, have neurogenic actions in hippocampus and PFC (32), our findings suggest that genes involved in the regulation of neurodevelopment or neurogenesis are candidate genes for treatment response in schizophrenics, as well as for schizophrenia per se.

PDE7B Is Candidate Gene Either for Treatment Response and Susceptibility for Schizophrenia

We pursued the top findings from Study 3 to see whether the findings with best convergent evidence (human and mouse) for relevance to risperidone response might also influence susceptibility to schizophrenia. After correction for multiple testing, we found evidence for association between disease status and *PDE7B*, which was therefore the only gene supported across all study designs.

Phosphodiesterases (PDEs) are central in regulating degradation of cAMP and cyclic guanosine monophosphate (cGMP), which are important second messengers for many cellular functions (33). There are 21 known genes encoding PDEs in human, spread across 11 distinct PDE families (*PDE1* to *PDE11*). Among these, *PDE4B* has been reported as a candidate susceptibility gene for schizophrenia. This was on the basis of a translocation found in two affected members of a single pedigree and the observation that the protein interacts with Disrupted in Schizophrenia 1 (*DISC1*), itself another strong candidate gene for schizophrenia and affective disorders (34). Elevation of cellular cAMP leads to dissociation of *PDE4B* from DISC1 and an increase in *PDE4B* activity (34).

PDE7B degrades cAMP, but not cyclic guanosine monophosphate (cGMP), and is predominantly expressed in brain (33). To date, no direct evidence for association of *PDE7B* with schizophrenia has been reported; however, several findings provide some functional plausibility to our results. First, mRNA for *PDE7B* and dopamine D1, D2, and D3 receptors show a similar pattern of distribution, and it is thought that the dopamine D1 receptor activates *PDE7B* through the cAMP pathway (35). Second, *PDE7B* maps to 6q23-24, one of the most significant linkage regions for schizophrenia (OMIM %600511; SCZD3) (36). Lastly, association has recently been reported to the *Abelson Helper Integration Site 1* (AHI1) and *Family with sequence similarity 54 A* (*FAM54A*), which are respectively in the 5′ and 3′ regions of *PDE7B*. However, it should be noted that in those studies, SNPs in *PDE7B* were not associated with disease (37,38).

PDE inhibitors have recently emerged as being of interest as therapeutic agents for neuropsychiatric disorders, such as schizophrenia, depression, and dementia (33). Our results indicate that among these, drugs acting on *PDE7B* may be of particular value in schizophrenia, although particularly for clinical applications, our results should be treated with caution until independent replications have been reported.

Limitations and Conclusion

The major limitation in this study is that the sample sizes we used for the genomewide pharmacogenetics and gene expression studies are small. In particular, the phramacogenetics study is only highly powered to detect effects that are much larger than typical of common susceptibility alleles for diseases to date. This is less of a limitation with respect to one major goal of pharmacogenetics, namely, the identification of common markers with sufficiently large effects to be of value in guiding therapeutics. Our study suggests that in such large common effects may not exist, although being based on one of the earliest chips, the coverage of genes is incomplete, and it would be desirable to repeat this experiment with a denser set of SNPs. The extent to which clinical heterogeneity is likely to have an impact on treatment response, and therefore power to detect association to that response, is also currently

unknown. We presume it is likely to play some role, as is the possibility of imperfect adherence to treatment. More subtle effects are of potential value in informing about drug mechanisms relevant to therapeutic response, and here, power is limited. Given that limitation, we tried to minimize false negatives through the use of relaxed significance criteria but tried to control false positives by combining expression and genetic data. Nevertheless, replication of our findings are required. Our follow-up observation of association between *PDE7B*, a novel candidate gene, and schizophrenia does, however, suggest that the use of convergent data may have successfully enriched for findings of true relevance to schizophrenia and its response to treatment.

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Supplementary material cited in this article is available online.

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Genome-Wide Association Study of Schizophrenia in a Japanese Population

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Background: Genome-wide association studies have detected a small number of weak but strongly supported schizophrenia risk alleles. Moreover, a substantial polygenic component to the disorder consisting of a large number of such alleles has been reported by the International Schizophrenia Consortium.

Method: We report a Japanese genome-wide association study of schizophrenia comprising 575 cases and 564 controls. We attempted to replicate 97 markers, representing a nonredundant panel of markers derived mainly from the top 150 findings, in up to three data sets totaling 1990 cases and 5389 controls. We then attempted to replicate the observation of a polygenic component to the disorder in the Japanese and to determine whether this overlaps that seen in UK populations.

Results: Single-locus analysis did not reveal genome-wide support for any locus in the genome-wide association study sample (best $p = 6.2 \times 10^{-6}$) or in the complete data set in which the best supported locus was *SULT6B1* (rs11895771: $p = 3.7 \times 10^{-5}$ in the meta-analysis). Of loci previously supported by genome-wide association studies, we obtained in the Japanese support for *NOTCH4* (rs2071287: $p_{meta} = 5.1 \times 10^{-5}$). Using the approach reported by the International Schizophrenia Consortium, we replicated the observation of a polygenic component to schizophrenia within the Japanese population ($p = .005$). Our trans Japan–UK analysis of schizophrenia also revealed a significant correlation (best $p = 7.0 \times 10^{-5}$) in the polygenic component across populations.

Conclusions: These results indicate a shared polygenic risk of schizophrenia between Japanese and Caucasian samples, although we did not detect unequivocal evidence for a novel susceptibility gene for schizophrenia.

Key Words: Genome-wide association study, *NOTCH4*, polygenic component, schizophrenia, *SULT6B1*

Epidemiologic studies show that genetic factors account for more than 80% of the population variance in susceptibility for schizophrenia; however, as with virtually all other relatively common disorders, it has historically proven difficult to identify the specific genetic variants involved (1).

The application of genome-wide association technology to large case–control samples of mainly European ancestry has recently implicated a number of risk loci for which the evidence is strong. These include loci defined by single nucleotide polymorphisms (SNPs) in which the effects are weak (odds ratios [ORs] 1.1–1.25) among which the strongest supported loci are *zinc finger protein 804 A* (*ZNF804A*) (2–5), a broad region including the major histocompatibility complex (MHC) on chromosome 6p21.3–22.1 (6–8), *neurogranin* (*NRGN*), and *transcription factor 4* (*TCF4*) (8).

Although the robust support for a number of recently implicated loci represents something of a break from the past inconsistencies, little of the genetic variance of schizophrenia can be explained by the loci identified thus far. One explanation for this is that much of the risk is conferred by common but weak genetic effects that require larger samples. Another explanation is that most of the risk cannot be readily detected by genome-wide association studies (GWAS), the missing genetic component being conferred by mutations that exert substantial individual effects that are rare or even unique to individual pedigrees.

Although the relative contributions of these classes of variant awaits empiric resolution, the GWAS of the International Schizophrenia Consortium (ISC) provided strong support for a substantial polygenic contribution (at least 30%) to the population risk of schizophrenia, much of which is conferred by common alleles with small effect sizes (6,9,10). The basic principle of their analysis was that in the presence of a substantial common polygenic component, although most of the individual genetic effects will not be

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detectable in current sample sizes, the sum of many such effects across multiple SNPs might differ between cases and controls. After discounting the influence of various potential sources of bias, the authors concluded that the findings were best explained by the existence of an important polygenic component to the disorder comprising a large number of common alleles, although some contribution from low-frequency alleles was not excluded or deemed unlikely (6).

There were two additional striking findings in the ISC article (6). The first was that those alleles selected as "risk" alleles for schizophrenia were also enriched in people with bipolar disorder, supporting the hypothesis of shared genetic susceptibility between these disorders (11,12). The second was that sets of "risk" alleles defined from white individuals of European origin were better at predicting affected status in other white European subjects than they were in African Americans, although an attenuated effect was seen in an African American sample. This may be attributable to differences in allele frequencies and linkage disequilibrium between Europeans and African Americans, although genetic heterogeneity remains a possibility. In this article describing a study that sought novel susceptibility variants, we report the first GWAS for schizophrenia in a Japanese sample. Although the Japanese population is considered relatively homogeneous (13), GWAS studies in other populations strongly suggest that our study of 575 cases and 564 controls is underpowered to detect any findings at genome-wide levels of significance. Thus, we attempted to enhance power by following up the top 150 of the most strongly supported SNPs from the GWAS in an independent sample of 1511 cases and 1517 controls drawn from the Japanese population as well as 479 cases and 2938 controls from the United Kingdom (2). We also sought to examine whether the Japanese population shares with Europeans a polygenic component for schizophrenia and bipolar disorder using schizophrenia and bipolar case–control samples from the United Kingdom that have been previously subjected to GWAS (2,14). Because it is unlikely that stratification effects would bias the allele distributions en masse in samples ascertained in Japan in the same direction as in a European sample, confirmation of a shared polygenic effect argues strongly against the idea that residual uncontrolled stratification is responsible for the effect. Moreover, because rare alleles of large effect are expected to reflect an ongoing process of new mutation (to compensate for their removal by selection), the existence of transcontinental effects also argue against the idea that rare alleles alone can drive this effect, it being unlikely that relatively new variants would be carried on the same ancestral haplotypes in both populations.

Methods and Materials

Participants

We selected 575 patients with schizophrenia (43.5 ± 14.8 years) and 564 healthy controls (44.0 ± 14.4 years) for genome-wide association analysis (our screening GWAS: [JPN_GWAS]). All subjects were unrelated, living in the Tokai area of the mainland of Japan, and self-identified as Japanese. The details of the sample and copy number variation analysis of this GWAS data set have been reported previously (15), and see also Supplement 1.

For follow-up studies, we used an independent Japanese sample comprising 1511 cases (aged 45.9 ± 14.0 years) and 1517 controls (aged 46.0 ± 14.6 years) diagnosed and ascertained in the same way as the GWAS data set. These samples were recruited from three areas on the Japanese mainland, comprising the Kansai and Chugoku areas in addition to the Tokai area. To enhance the sample in the replication analysis, data were added from 934 Japanese controls genotyped by Illumina550 (Illumina, San Diego, California) as part of the Japanese Single Nucleotide Polymorphisms (JSNP) project (http://snp.ims.u-tokyo.ac.jp/index.html). If SNP data were available in the JSNP sample, we merged the two sample sets to form a final Japanese replication sample (we refer this as "Rep_JPN") comprising 1511 cases and 2451 controls (SNPs genotyped in both samples can be seen in Table S1 in Supplement 2).

We additionally included data from a UK schizophrenia GWAS data set of 479 cases and 2938 controls genotyped using the Affymetrix 500K array (Santa Clara, California), details of which have been reported before (2,14).

For the polygenic component analysis, we also included the Wellcome Trust Case-Control Consortium (WTCCC) bipolar disorder data set of 1868 cases and 2938 shared controls, details of which are reported elsewhere (2,14).

After complete description of the study to the subjects, written informed consent was obtained. This study was approved by the ethics committees of each university participating in this project.

GWAS and Quality Control

Genotyping was performed using the Affymetrix Genome-Wide Human SNP Array 5.0 according to the manufacturer's protocol. After applying several quality control (QC) criteria (e.g., call rate ≥ 95%, autosomal chromosomes, Hardy–Weinberg equilibrium (HWE) ≥ .0001 and minor allele frequency [MAF] ≥ 5%; Supplement 1), the final GWAS consisted of 1108 samples (560 cases and 548 controls) and 297,645 SNPs (MAF ≥ 5%).

Q-Q plots were generated on the basis of allele-wise analysis of SNPs that passed QC (Supplement 1), and our observed value of λ is consistent with those generally reported in well-matched samples ($\lambda = 1.065$ and $\lambda_{1000} = 1.117$).

Follow-Up Genotyping

Follow-up genotyping in our independent Japanese case–control sample was performed by Sequenom (San Diego, California) using the Sequenom iPLEX Gold System. Markers that could not be assayed on this platform were genotyped using a TaqMan assay (Applied Biosystems, Foster City, California).

Candidate SNPs were selected for replication as follows. First, the top 200 SNPs were identified (corresponding to $p \sim < 5 \times 10^{-4}$). Highly correlated markers based on $r^2 > .9$ to a more significant marker within 100 kb (r^2 was based on HapMap information [release Number 24, October 2008] and our own GWAS from controls) were then removed. From this list, we included the following: 1) SNPs with $p < 5 \times 10^{-5}$ ($n = 15$ after 11 redundant SNPs removed. Total number = 26. Of these, two SNPs failed for primer design. 2) Under the premise that in GWAS analysis, power favors more common alleles and that the enrichment for true associations is greater in this category of alleles (6), SNPs with MAF ≥ 10% surpassing a more relaxed threshold ($P < \sim 3.5 \times 10^{-4}$) were selected, corresponding to the top 150 SNPs ($n = 76$ after 12 low MAF SNPs and 36 redundant SNPs removed. This resulted in a total of 124. Of these, 5 SNPs failed primer design. We additionally included 13 SNPs that ranked from 151st to 200th on the grounds that they could be included in the Sequenom panels of markers without compromising the design of the higher-priority SNPs. Consequently, 97 SNPs were genotyped in the replication sample, of which 5 did not pass QC on the basis of genotype call rate (> .95) and HWE ($p > .001$). All genotype calls were confirmed by visual inspection of cluster plots.

SNP-Based Association Analysis

Consistent with most other GWAS, our study is based upon allele-wise association analysis which assumes an additive model.

Genomic control adjusted p values were also calculated based upon median chi-square statistics. This was performed using PLINK v1.07 (16).

Combined analysis across data sets (Meta_JPN: JPN_GWAS + Rep_JPN, Meta_ALL: JPN_GWAS + Rep_JPN + UK schizophrenia) were conducted using the Cochran–Mantel Haenszel (CMH) approach conditioned by sample as implemented in PLINK v. 1.07.

Polygenic Component Analysis

Discovery (for selecting "score alleles" based on association statistics) and targeting (for calculation of polygenic score) samples are summarized in Table S2 in Supplement 1. Briefly, we examined five discovery and target pairs:

1. Japanese: A set of 280 cases and 274 controls were selected for discovery, and the results were tested in an additional set of 280 cases and 274 controls. The discovery/target samples were selected at random (on the basis of random number generation) from the Japanese GWAS data set. This procedure was repeated 1000 times to ensure the results of this analysis were representative of random divisions of the data set.

2, 3. Each of the UK schizophrenia (479 schizophrenia and 2938 controls) (2) and bipolar (1868 cases and 2938 controls) (14) samples were used separately as a discovery data set to generate lists of "risk" alleles that were tested in the full Japanese GWAS sample.

4, 5. The full Japanese GWAS sample was used as a discovery data set to generate lists of "risk" alleles that were tested in the UK schizophrenia and bipolar data sets.

For the UK data sets, we used the QC criteria applied in the primary manuscripts (2,14) in which SNPs that deviated from HWE ($p < 1 \times 10^{-5}$ in cases or .001 in control) and had a low call rate (< 97%) were excluded. Note that the criteria for HWE exclusion in the UK data set is slightly different from that in the Japanese GWAS. The precise choice of HWE filter is arbitrary, but we note that both data sets criteria are on the more stringent side of customary practice.

Following the ISC (6), we reduced the set of SNPs by removing SNPs that are in linkage disequilibrium (LD) using the same criteria applied by the ISC (r^2 threshold at .25, window size 200 SNPs). In the tests of the split Japanese data set, we used LD-pruned SNPs selected on the basis of the metrics in the full set of Japanese controls. For all comparisons between Japanese and European data sets, we pruned SNPs sequentially first on the basis of the LD metrics in the discovery data set and second on those in the target data set. Polygenic score was calculated by weighting scores for "risk" alleles by the logOR observed in the discovery data set according to the method used by the ISC (6).

Nominally associated alleles were selected on the basis of the genomic-control adjusted p value in the allele-wise association analysis from the discovery samples at the following liberal significance thresholds (P_T) ($P_T < .5$, $P_T < .4$, $P_T < .3$, $P_T < .2$ and $P_T < .1$). The polygenic score was calculated using PLINK v. 1.07. Nagaelkerke's pseudo R^2 (a measure of variance explained by a particular factor) was calculated by logistic regression analysis using *R* (http://www.r-project.org) with covariation for "nonmissing SNPs" according to the ISC study (6).

Results

Single Marker Association Analysis

A summary plot of the GWAS (MAF ≥ 5%) is presented in Figure S1 in Supplement 1. We did not observe any associations at a widely used approximate benchmark for genome-wide significance ($p = 7.2 \times 10^{-8}$) (17). The strongest associations were observed at rs12218361, which maps to chromosome 10 at 126.06 Mb and is 3′ of *ornithine aminotransferase* (*OAT*, $p_{allele} = 6.2 \times 10^{-6}$, two-tailed), and rs11895771, which maps to chromosome 2 at 37.27 Mb within *sulfotransferase family, cytosolic, 6 B, member1* (*SULT6B1*, $p_{allele} = 8.0 \times 10^{-6}$, two-tailed). The most significant 200 markers are given in Table S1 in Supplement 2.

We genotyped 97 LD-pruned SNPs mainly from the top 150 GWAS findings in an independent Japanese replication sample (1511 cases and 1517 controls). For 22 of these, it was possible to expand the control sample size using data from the Japanese population based on the public database (JSNP). Data for 81 SNPs were also available in the UK data set (Affymetrix 500 K chip) and were included in the association analysis. On the basis of the replication sample from Japanese (Rep_JPN) alone, rs9880957 showed the most significant association ($p = 2.8 \times 10^{-3}$, two-tailed, OR = 1.2), but the associated allele was not the same as in the GWAS. Additionally, we undertook set-based analysis (using PLINK) to investigate whether there was an excess of association signals for these top GWAS findings in the replication data set that surpassed nominal p thresholds (e.g., $p < .1, .05, .01, .001$) in the Rep_JPN and UK data sets (10,000 permutation without lambda correction for all SNPs that passed the p threshold). However, no significant enrichment was observed (data not shown). That finding is compatible with the polygenic analysis we describe subsequently and with the now widely accepted hypothesis that common alleles that might be detectable in principle by GWAS exert effects that are too weak to be substantially enriched for associations that surpassed the threshold we specified for follow-up.

In the CMH analysis of the complete Japanese sample (Meta_JPN: JPN_GWAS + Rep_JPN), the best p was found at rs1011131 in LOC392288 ($p = 1.2 \times 10^{-4}$, two-tailed), which is weaker than in the initial GWAS ($p = 2.5 \times 10^{-5}$, two-tailed). Further expanding the sample size by including UK samples (Meta-ALL: JPN_GWAS + Rep–JPN + UK schizophrenia) did not provide convincing support for any locus (Table S1 in Supplement 2). The strongest association signal in Meta_ALL was rs11895771 ($p = 3.7 \times 10^{-5}$, two-tailed) in *SULT6B1*, which had been ranked second in the screening GWAS (Table 1).

Excluding *ZNF804A* (the Japanese data for which were included in the paper by O'Donovan *et al.*) (2), we additionally tested regions containing schizophrenia candidate loci supported by genome-wide significant associations in previous GWAS data sets (6–8). Specifically, we focused on three regions: the MHC region (Chr6 25 ~ 33 Mb), *NRGN*, and *TCF4*. In this analysis, we first imputed ungenotyped SNPs in these regions (boundaries ± 1 Mb) for fine mapping (the imputation method is presented in Supplement 1). None of the specific SNPs at these loci that have been reported by others (6–8) as genome-wide significant were imputable in our Japanese GWAS sample (Figures S2–S4 in Supplement 1). However, interestingly, we did observe a strong, fairly well circumscribed association signal on chromosome 6 in the region of *NOTCH4* (Figure S2 in Supplement 1). Furthermore, genetic association within *NOTCH4* has been reported (18) in another Japanese study (non-overlapping with the present sample) at rs2071287 (Figure S2 in Supplement 1), which is in complete LD ($D' = 1$, $r^2 = .56$) with rs2071286, the best SNP tested in our GWAS data. Because that previously supported SNP (rs2071287) is also associated in our GWAS ($p = 2.1 \times 10^{-3}$), we then followed up this SNP in the Rep_JPN sample; rs2071287 was again significantly associated ($P_{allele} = .018$, two-tailed, Figure S5 in Supplement 1; note: we could not impute this SNP with high confidence in the UK schizophrenia