

**Table 5**

Haplotype analyses for the SNPs rs5756154–rs739096 in *MYH9* in the screening, confirmatory and combined populations.

Haplotype	Patients	Controls	<i>p</i>
Expanded screening population			0.023 <sup>a</sup>
1–1	0.844	0.878	0.018
2–2	0.146	0.115	0.024
Confirmatory population			0.518 <sup>a</sup>
1–1	0.838	0.842	0.627
2–2	0.114	0.119	0.413
Combined population			0.619 <sup>a</sup>
1–1	0.840	0.849	0.149
2–2	0.120	0.118	0.735

Major and minor alleles are denoted by 1 and 2, respectively.

<sup>a</sup> Global *p* values.

second stage and those in the third stage. Allelic heterogeneity may exist for *MYH9*. In this case, it would be difficult to provide convincing evidence for an association. It might be noteworthy that a recent genome-wide association study (GWAS) of major depressive disorder suggested that there may be allelic heterogeneity for *glutamate receptor, metabotropic 7 (GRM7)* (Muglia et al., 2008). Nevertheless, our results could not show sufficient evidence for an association of *MYH9* with schizophrenia in the Japanese population.

The genetic variants of *MYH9* have previously been tested for associations with schizophrenia. An initial study showed significantly distorted transmission of AFM262VH5 in 23 families multiply affected with schizophrenia (Vallada et al., 1995). However, subsequent case-control studies failed to replicate this association (Kitao et al., 2000; Williams et al., 1997). Our three-stage case-control study could not provide sufficient evidence for an association of *MYH9* with schizophrenia. It is noteworthy that although there were no significant associations between seven SNPs in *MYH9* and schizophrenia, four SNPs (rs3752463 in intron 9, rs1557540 in intron 3, rs713839 in intron 3, and rs739097 in intron 1) in *MYH9* were associated with a subgroup of schizophrenia patients without deficits in sustained attention (Liu et al., 2008). Considering these findings together, *MYH9* does not contribute to genetic susceptibility to schizophrenia, but may have effects on the neuropsychological endophenotypes for schizophrenia.

We recognize some limitations of the present study. First, we screened only 12 microsatellite markers with an average inter-marker interval of 2.87 Mb. Therefore, our results cannot exclude possible contributions of other genes on 22q to the pathogenesis of schizophrenia. Recent studies using large samples (more than 1000 cases and 1000 controls) have indicated that genes on 22q including *protein interacting with PRKCA 1 (PICK1)*, *claudin 5 (CLDN5)*, *DiGeorge syndrome critical region gene 2 (DGCR2)*, *armadillo repeat gene deletes in velocardiofacial syndrome (ARVCF)* and *COMT* are not associated with schizophrenia (Ishiguro et al., 2007, 2008a,b; Okochi et al., 2009; Sanders et al. 2008). However, to draw a definitive conclusion, further studies using large samples and sufficient markers should be carried out in various ethnic populations. Several GWAS of schizophrenia have been published (International Schizophrenia Consortium, 2009; Kirov et al., 2009; Lencz et al., 2007; Mah et al., 2006; Need et al., 2009; O'Donovan et al., 2008; Shi et al., 2009; Shifman et al., 2008; Stefansson et

al., 2009; Sullivan et al., 2008). Interestingly, a polymorphism in intron 1 of *myosin XVIII B (MYO18B)* on 22q12.1 was most significantly associated with schizophrenia in a large-scale GWAS (International Schizophrenia Consortium, 2009). A meta-analysis of GWAS for schizophrenia, which is currently being conducted (Psychiatric GWAS Consortium Coordinating Committee, 2009), will be useful for the process of narrowing down the region for fine mapping on 22q. Second, our individuals were not assessed using a standardized structured interview. However, the diagnosis of schizophrenia was made on the basis of all available sources of information. To the best of our knowledge, there were no control individuals who were likely to develop schizophrenia at their present stage of life. Thus, it is unlikely that our failure to find a significant association is attributable to misdiagnosis. Despite these caveats, our results suggest that *MYH9* does not confer increased susceptibility to schizophrenia in the Japanese population.

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#### Contributors

Authors Amagane and Watanabe designed the study and performed genotyping. Authors Kaneko and Nunokawa undertook statistical analyses. Author Muratake designed the study. Author Ishiguro performed genotyping. Authors Arinami, Ujike, Inada, Iwata, Kunugi, Sasaki, Hashimoto, Itokawa, and Ozaki managed sample collection. Author Someya supervised the study. All authors contributed to and have approved the final manuscript.

#### Conflict of interest

None of the authors has a conflict of interest to declare.

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## Letter to the Editor

**No association between *DAO* and schizophrenia in a Japanese patient population: A multicenter replication study**

Dear Editors

D-serine, a co-agonist that enhances N-methyl-D-aspartate (NMDA) glutamate receptors, is hypothesized to be involved in the pathophysiology of schizophrenia because treatment with D-serine improves some schizophrenic symptoms (Tsai et al., 1998). Thus, genes that are related to D-serine metabolism have arisen as candidate genes that may play a role in schizophrenia. Genomic case-control studies of one such candidate gene, D-amino acid-oxidase (*DAO*; 12q24), have shown relatively consistent positive results in which *DAO* is a susceptibility locus for patients with schizophrenia (Chumakov et al., 2002; Corvin et al., 2007; Schumacher et al., 2004; Wood et al., 2007). A previous well-explained report about the role of *DAO* in schizophrenia (Verrall et al., 2009) and two representative meta-analyses showed an association between *DAO* and schizophrenia, specifically with the SNP, rs4623951 (Allen et al., 2008; Shi et al., 2008). Previously, we performed stage 1 genomic case-control studies (340 schizophrenia patients, 340 healthy controls) for genes (*PHCDH*, *SHMT1*, *SRR*, *DAO*) related to D-serine synthesis/degradation. Our results from a haplotype case-control analysis showed that only three SNPs (rs3825251, rs3918347, and rs4964770) in *DAO* which are in strong linkage disequilibrium (LD) and show an association with schizophrenia, even after correction for multiple testing (Ohnuma et al., 2009). Some negative results have also been reported regarding a possible association between *DAO* and schizophrenia (Fallin et al., 2005; Jonsson et al., 2009; Liu et al., 2006; Shinkai et al., 2007; Vilella et al., 2008; Yamada et al., 2005). Thus, we performed a stage 2 replication study to clarify the conclusions of a possible role for *DAO* in schizophrenia. We reinvestigated the association between the above *DAO* haplotype (rs3825251–rs3918347–rs4964770) and schizophrenia using adequate statistical power.

The case-control genetic association studies were performed using 1656 unrelated Japanese patients with schizophrenia (863 males, 793 females; mean age 45.2 years, S.D.  $\pm$  15.4) who met the DSM-IV diagnosis of schizophrenia. We also used 1842 unrelated healthy controls (784 males, 1058 females; mean age 47.1 years, S.D.  $\pm$  19.1). Patients and controls were recruited from four geographic regions of Japan: Osaka, Aichi, Saitama, and Tokyo. The criteria for

enrolling both patients and controls have been previously described (Ohnuma et al., 2009). Of these participants, 340 patients with schizophrenia and 340 healthy controls had been analyzed in our previous stage 1 study (Ohnuma et al., 2009); the remaining patients and controls were new to the present study. The mean age of the schizophrenic group was significantly different from that of the control group (Student's *t*-test,  $t = 3.27$ ,  $P = 0.001$ ), and the gender distribution of the two groups was significantly different ( $\chi^2 = 31.9$ ,  $P < 0.001$ ). The Ethics Committee of the Juntendo University School of Medicine approved this study. All participants gave their written informed consent prior to participating in the study.

Genomic DNA was extracted from peripheral white blood cells using a QIAamp® DNA Blood Maxi kit (Qiagen, Courtaboeuf, France). Three SNPs (rs3825251, rs3918347, and rs4964770) were typed using TaqMan® technology as previously described in detail (Ohnuma et al., 2009). SNP information, including position and distance between SNPs, has also been described (Ohnuma et al., 2009).

For the case-control association study, genetic statistical analysis, Hardy–Weinberg Equilibrium (HWE) testing, differences in genotypic/allelic frequencies, LD, and case-control haplotype analysis were all done using SNPAllyse V7.0 Pro (Ohnuma et al., 2009). All *P*-values reported are two-tailed and were considered statistically significant when  $< 0.05$ . We performed power calculations using the Power Calculator for Two Stage Association Studies (<http://www.sph.umich.edu/csg/abecasis/CaTS/>). Power was calculated under the prevalence of 0.01 using an additive or a multiplicative model, which was based on allelic frequencies of the associated markers that ranged from 0.49 (rs3918347) to 0.50 (rs4964770). The odds ratio ranged from 1.152 (rs3918347) to 1.166 (rs4964770) for the SNPs investigated in our current study (Ohnuma et al., 2009), with an alpha level of 0.05. Results of power analysis showed that the power ranged from 80% (rs3918347) to 90% (rs4964770).

The three SNPs in *DAO* were genotyped in 1656 patients with schizophrenia and in 1842 controls. No deviation from HWE in either patients or controls was observed, and no single SNP showed a significant association between their allelic or genotypic frequencies and schizophrenia (Supplementary Table 1). Further investigation of the three SNPs showed a strong LD (Table 1), but case-control haplotype association analysis (minor haplotypes with frequencies less than 3% in either group were omitted) using windows of two or three SNPs failed to show a significant association with Japanese schizophrenia (Table 1).

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**Table 1**

Results of linkage disequilibrium ( $D'/r^2$  value) between the SNPs and two- and three-SNP-based haplotype analyses.

SNPs	$D'/r^2$ <sup>a</sup>	Haplotypic global P-value	
		2 windows	3 windows
rs3825251	0.91/0.82	0.93/0.86	0.85
rs3918347	0.96/0.89	0.95/0.88	0.87
rs4964770	0.94/0.87	0.98/0.97	

<sup>a</sup> For  $D'/r^2$  value, right upper diagonal: schizophrenia, left lower diagonal: controls.

In our previous stage 1 study, the 3' regions of *DAO* showed a strong LD, including three SNPs (rs3825251–rs3918347–rs4964770) that showed an association with Japanese schizophrenia. In particular, haplotype GGC may be a protective haplotype in schizophrenia (Ohnuma et al., 2009). But in our current stage 2 study that had adequate statistical power, our results did not show any significant association between schizophrenia and single SNPs or two- and three-SNP-based haplotypes. Thus, the results from our previous study should be considered a type I error (false positive), because they did not have adequate statistical power to produce reliable results. Previously, two representative meta-analyses showed an association between rs4623951 in the 5' region and schizophrenia; however, none of the studies that only used Japanese subjects showed positive findings for rs4623951, whereas the studies with only Japanese subjects showed marginal positive findings in the 3' region (Ohnuma et al., 2009; Yamada et al., 2005). In addition, Japanese subjects were not included in all studies that reported positive associations between schizophrenia and SNPs situated in the 5' region of *DAO* (Chumakov et al., 2002; Corvin et al., 2007; Schumacher et al., 2004; Wood et al., 2007). Taken together, these results suggest that there may be some ethnic differences in *DAO*, and that a schizophrenia susceptibility locus in Japanese patients may have a nominal effect and may be situated in the 3' region. Indeed, the minor allelic frequencies of the three investigated SNPs, especially rs3825251, were different among different ethnic populations, and the LD pattern in the 3' region was also different between Japanese and Caucasian individuals (<http://www.hapmap.org/index.html>). In addition, symptomatic differences and schizophrenia subtypes were not considered in the current study, and thus the heterogeneity of this disease may also lead to inconsistent results in genetic studies of schizophrenia.

Thus, further genetic case-control studies of *DAO* with careful consideration of schizophrenia heterogeneities (i.e., differences in ethnicity, clinical subtypes, and symptoms) and investigation of gene–gene interactions among D-serine-related genes may be needed to determine a possible association with schizophrenia. Thus, we currently conclude that *DAO* does not have an apparent degree of association with Japanese schizophrenia.

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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.2010.01.028.

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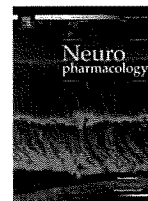
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## Association analysis of *SIGMAR1* with major depressive disorder and SSRI response

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## SUMMARY

**Background:** Several investigations have suggested the possible involvement of sigma1 non-opioid intracellular receptor 1 (sigma1 receptor) in the pathophysiology of major depressive disorder (MDD). Sigma1 receptors are also one of the major pharmacological therapeutic targets of selective serotonin reuptake inhibitors (SSRIs). To evaluate the association of sigma1 receptor gene (*SIGMAR1*) and MDD and SSRIs therapeutic response in MDD, we conducted a case–control study of Japanese samples (466 MDD patients, 516 controls and 208 MDD patients treated by fluvoxamine or sertraline).

**Method:** We defined a clinical response as a decrease of more than 50% in baseline the Structured Interview Guide for Hamilton Rating Scale for Depression (SIGH-D) within 8 weeks, and clinical remission as an SIGH-D score of less than 7 at 8 weeks. Therefore, we selected rs1800866 in *SIGMAR1* for the following association analysis.

**Results:** In the logistic regression analysis, we detected an association of the phenotypes (MDD or controls) with rs1800866 genotype. However, we did not detect an association between rs1800866 and SSRI therapeutic response in Japanese MDD. In addition, remission with SSRI was not associated with rs1800866. Also, we did not detect a novel polymorphism in *SIGMAR1* when we performed a mutation search using MDD treated by SSRIs samples.

**Conclusion:** Our results suggest that rs1800866 in *SIGMAR1* may play a role in the pathophysiology of MDD in the Japanese population. Also, *SIGMAR1* does not play a role in the therapeutic response to SSRI in Japanese MDD patients. However, because our sample was small, a replication study using another population and larger sample will be required for conclusive results.

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### 1. Introduction

Several investigations have suggested the possible involvement of sigma1 non-opioid intracellular receptor 1 (sigma1 receptor) in the pathophysiology of major depressive disorder (MDD) and the pharmacological therapeutic response in MDD (Bermack and Debonnel, 2005; Hashimoto and Ishiwata, 2006; Hayashi and Su, 2008; Ishikawa et al., 2007; Kulkarni and Dhir, 2009).

Preclinical studies suggest that fluvoxamine and sertraline, which are selective serotonin reuptake inhibitors (SSRIs), have

effects on sigma1 receptors (Ishikawa et al., 2007; Narita et al., 1996). The sigma1 receptor is more abundant in the dentate gyrus of the hippocampal formation, facial nucleus and various thalamic and hypothalamic nuclei (Bouchard and Quirion, 1997). Sigma1 receptors regulate dopamine release in various areas of the brain (Gonzalez-Alvear et al., 1995), and regulate dopaminergic, N-methyl-D-aspartate (NMDA) and glutamatergic neurotransmission in limbic areas, including the nucleus accumbens and the ventral tegmental area (Ault and Werling, 1999; Gronier and Debonnel, 1999; Weatherspoon et al., 1996; Yamazaki et al., 2002). These mechanisms are known to be involved in the pathophysiology of MDD (Miller et al., 2009; Nestler and Carlezon, 2006). Neurodevelopmental and neurodegenerative models have been suggested to be involved in other aspects of the pathophysiology of

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MDD (Ansorge et al., 2007). The hippocampal volume in MDD patients is significantly smaller than in control subjects (Kronmuller et al., 2009). Wang and Duncan reported that both neural survival and growth pathways are activated and the caspase-3-dependent apoptosis pathway is inactivated when sigma1 receptors are functionally active (Wang and Duncan, 2006). Nishimura and colleagues reported that sigma1 receptor agonist is involved in the mechanisms causing the drugs' potentiation of nerve growth factor (NGF)-induced neurite outgrowth in PC12 cells (Nishimura et al., 2008). Moreover, sigma1 receptors were shown to interact with inositol 1,4,5-triphosphate (IP3) receptors (Nishimura et al., 2008). Sigma1 receptor knockout mice also showed a depressive-like phenotype (Sabino et al., 2009). On the other hand, sigma1 receptors were reported to play an important role in the pathogenesis of anxiety and response to stress (Kamei et al., 1998; Lambert et al., 1998; Maurice et al., 1999), and depression (Akunne et al., 1997; Bermack and Debonnel, 2001).

From this evidence, we thought that the sigma1 receptor gene (*SIGMAR1*: OMIM \*601978, 2 exons in this genomic region spanning 3.850 kb, and located on 9p13) was a good candidate gene for the pathophysiology of MDD. Several genome-wide association studies reported no association between *SIGMAR1* and MDD (Muglia et al., 2008; Sullivan et al., 2009) or clinical response to treatment with antidepressants in MDD (Garriock et al., 2010; Laje et al., 2009). However, we found no genetic association study that used Japanese samples. To evaluate the association of *SIGMAR1* and MDD and SSRIs therapeutic response in MDD, we conducted a case-control study of Japanese samples (466 MDD patients, 516 controls and 208 MDD patients treated by SSRIs).

## 2. Materials and methods

### 2.1. Subjects

The subjects in the association analysis were 466 MDD patients (218 males and 248 females; mean age  $\pm$  standard deviation  $48.7 \pm 16.0$  years) and 516 healthy controls (266 males and 250 females;  $39.4 \pm 17.1$  years). The patients were diagnosed according to DSM-IV criteria with the consensus of at least two experienced psychiatrists on the basis of unstructured interviews and a review of medical records. 265 of the 466 MDD patients, who were treated with fluvoxamine (136 patients), sertraline (72 patients) or paroxetine (57 patients), were diagnosed according to DSM-IV criteria with the consensus of at least two experienced psychiatrists on the basis of a review of medical records and assessments with the Structured Interview Guide for Hamilton Rating Scale for Depression (SIGH-D) (Williams, 1988). Our subjects were hospital in-patients or outpatients, unrelated to each other and ethnically Japanese; cases were collected between January 2000 and December 2009. Almost patients were treated in the Department of Psychiatry,

Fujita Health University Hospital, and University of Occupational and Environmental Health, Japan. The healthy controls were psychiatrically screened based on unstructured interviews. None had severe medical complications such as liver cirrhosis, renal failure, heart failure or other Axis-I disorders according to DSM-IV. No structured methods were used to assess psychiatric symptoms in the controls, which included hospital staff and medical students. The healthy controls were also unrelated to each other and ethnically Japanese, enrolled between January 2000 and December 2009. The study was described to subjects and written informed consent was obtained from each. This study was approved by the Ethics Committees at Fujita Health University, Nagoya University Graduate School of Medicine and the University of Occupational and Environmental Health.

### 2.2. Data collection

Fluvoxamine and sertraline affect on sigma1 receptors (Ishikawa et al., 2007; Narita et al., 1996). Therefore, we performed a pharmacogenetic study using 208 MDD patients treated with fluvoxamine or sertraline. The 208 MDD patients in this study had scores of 12 or higher on the 17 items of the SIGH-D (Peveler and Kendrick, 2005). We defined a clinical response as a decrease of more than 50% in baseline SIGH-D within 8 weeks, and clinical remission as a SIGH-D score of less than 7 at 8 weeks. Fluvoxamine and sertraline were increased gradually to a maximum of 150 mg and 100 mg, respectively, depending on the patient's condition. Patients with insomnia and severe anxiety were prescribed benzodiazepine drugs, but no other psychotropic drugs were permitted during the study. Detailed information on data collection was described in a previous paper (Saito et al., 2006). The clinical characteristics of patients in this study, classified according to these definitions, can be seen in Table 1.

### 2.3. SNP selection and LD evaluation

We first consulted the HapMap database (release#24/phase II, Nov 2008, www.hapmap.org, population: Japanese Tokyo, minor allele frequencies (MAFs) of more than 0.05) and included one SNP (rs10814130) covering *SIGMAR1*. However, we thought that information of only one SNP on *SIGMAR1* from the HapMap database would be insufficient to reflect the detailed linkage disequilibrium (LD) background in the Japanese population. Rs1799729 and rs1800866 were reported to possibly be biologically functional SNPs (Prasad et al., 1998; Seth et al., 1997). Uchida and colleagues reported that LD from rs1800866 and rs1799729 was very tight ( $r^2 = 1.00$ ) (Fig. 1) (Uchida et al., 2003). Therefore, we selected rs1800866 for LD evaluation. In order to reflect the LD background in the Japanese population, we genotyped rs1800866, which was reported to have a substitution from glutamine (CAG) to proline (CCG) that may perturb appropriate regulation of transportation from the endoplasmic reticulum to the plasma membrane of sigma1 receptors (Seth et al., 1997), and rs10814130 using our 40 of our own control samples, and made a more detailed LD evaluation of *SIGMAR1*. We determined the LD between rs1800866 and rs10814130 using HAPLOVIEW software (Barrett et al., 2005). Because LD from rs1800866 to rs10814130 was very tight in our control samples ( $r^2$  more than 0.923) (Fig. 1), we selected rs1800866 for the following association analysis.

### 2.4. SNP genotyping

We used TaqMan assays (ABI: Applied Biosystems, Inc., Foster City, CA,) for rs1800866. One allelic probe was labeled with FAM dye and the other with fluorescent VIC dye. The plates were heated for 2 min at 50 °C and 95 °C for 10 min,

**Table 1**  
Clinical characteristics of the patients in both definition groups.

	N			Patients permitted with SSRIs, n <sup>c</sup>		Age, (mean $\pm$ SD)	Baseline SIGH-D, (avg $\pm$ SD)	SSRIs dose at eight weeks <sup>d</sup>	Number of previous episode, (avg $\pm$ SD)	Patients permitted with anxiolytics/hypnoticse, n (%)
	Total	Male	Female	FLV	STL					
Overall	208	100	108	136	72	47.8 $\pm$ 16.8	20.1 $\pm$ 5.48	119 $\pm$ 39.7	1.89 $\pm$ 0.818	69 (32.4)
<i>Clinical response group<sup>a</sup></i>										
Responders	117	52	65	70	47	47.5 $\pm$ 15.9	21.1 $\pm$ 5.46	118 $\pm$ 37.9	1.83 $\pm$ 0.781	45 (21.1)
Nonresponders	91	48	43	66	25	48.4 $\pm$ 18.0	18.8 $\pm$ 5.11	122 $\pm$ 42.2	2.02 $\pm$ 0.871	24 (11.3)
P-value	0.249			0.0862		0.694	<b>0.00140</b>	0.462	0.179	0.103
<i>Clinical remission group<sup>b</sup></i>										
Remitters	86	40	46	54	32	46.9 $\pm$ 15.8	19.7 $\pm$ 4.60	113 $\pm$ 39.6	1.94 $\pm$ 0.716	30 (14.1)
Nonremitters	122	60	62	82	40	48.6 $\pm$ 17.5	20.5 $\pm$ 5.98	124 $\pm$ 39.2	2.04 $\pm$ 0.878	39 (18.3)
P-value	0.814			0.397		0.499	0.291	<b>0.0462</b>	0.112	0.878

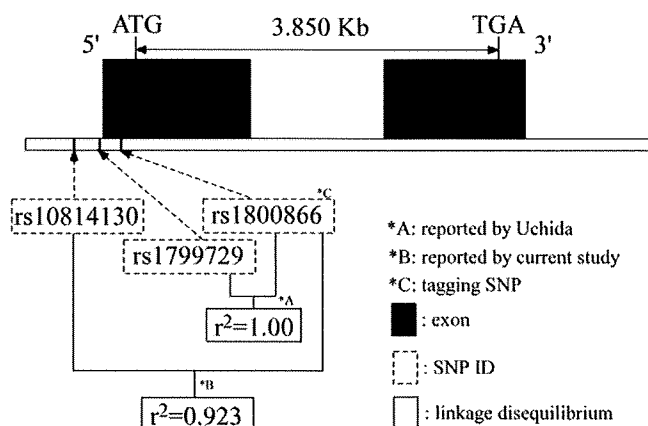
<sup>a</sup> Clinical response was defined as a 50% or greater decrease in the baseline SIGH-D score.

<sup>b</sup> Clinical remission was defined as a final SIGH-D score of less than 7.

<sup>c</sup> FLV: Fluvoxamine and STL: Sertraline.

<sup>d</sup> Imipramine-equivalent.





**Fig. 1.** LD evaluation in *SIGMAR1*. ATG is the start codon and TAG is the stop codon. Vertical bars represent exons. SNPs in *SIGMAR1*, which were already known, are represented by black boxes. The  $r^2$  value is represented by broken line boxes. \*A: reported by Uchida. \*B: reported by current study. \*C: tagging SNP.

followed by 45 cycles of 95 °C for 15 s and 58 °C for 1 min. Please refer to ABI for the primer sequence. We also used direct-sequence for rs10814130. Detailed information is available on request.

2.5. Mutation screening

We detected a significant association between *SIGMAR1* and MDD. In addition, therapeutic response and remission with fluvoxamine or sertraline was also marginally associated with rs1800866. Therefore, we performed mutation screening with *SIGMAR1* (promoter region, all exons including branch site) in 32 MDD treated by SSRIs patients (16 males and 16 females) using the primer extension method. We randomly selected 16 MDD patients of each sex after dividing by sex. The PCR method, including primer sequence and PCR condition, was adopted with reference to the study of Uchida and colleagues (Uchida et al., 2003). More detailed information about the primer extension method were published elsewhere (Suzuki et al., 2003).

2.6. Statistical analysis

Genotype deviation from the Hardy-Weinberg equilibrium (HWE) was evaluated by chi-square test (SAS/Genetics, release 8.2, SAS Japan Inc, Tokyo, Japan). The distribution of patient characteristics between MDD group and healthy control group was analyzed using a t test or a chi-square test. We found a significant difference in age among these groups ( $P_{age} < 0.0001$ ), however, there was no difference in gender distribution ( $P_{gender} = 0.339$ ). Although we investigated the difference in rs1800866 MAF by gender in the MDD cases, we did not detect a significant difference between MAF in males (0.257) and females (0.262). However, the number of male MDD patients was larger than the number of male healthy controls. We therefore performed a logistic regression analysis to compare the phenotype among rs1800866 genotypes to adjust possible confounding. The phenotype (MDD or controls) was the dependent variable, and gender, age at the time of recruitment and rs1800866 genotype were set as the independent variables. In the pharmacogenic study, individual t test and chi-square tests were used to compare means and categorical proportions (responders or nonresponders and remitters or nonremitters), respectively. Among the clinical characteristics of the patients in this study, a significant difference with total SIGH-D score was detected at the baseline in relation to SSRI therapeutic response ( $P$ -value = 0.00140) (Table 1). We also found another significant difference in SSRI dose at eight weeks between remitters and nonremitters ( $P$ -value = 0.0462) (Table 1). Therefore, we performed an analysis of the possible correlations between response or remission, SSRI treatment, and several clinical factors, using logistic regression. In these analyses, response classification was set as the dependent variable, and gender, age at the time of recruitment, SSRIs dose at eight weeks (imipramine-equivalent), SIGH-D total score at the baseline, and rs1800866 genotype were set as the independent variables. Polymorphisms were categorized into three genotypes, homozygous wild-type, heterozygous, and homozygous variant. Homozygosity for the more common allele was treated as the reference category. Tests for linear trend of odds ratio (additive model) were calculated using an ordered categorical variable by assigning scores to the genotypes: 0 (no variant allele), 1 (carrying one variant allele), and 2 (carrying two variant alleles). The statistical package JMP for Windows was used for logistic regression analysis (JMP 5.0.1J, SAS Japan Inc, Tokyo, Japan).

Power calculation was performed using a Genetic Power Calculator (Purcell et al., 2003). We set each item in each value in the Genetic Power Calculator as follows: Prevalence: 0.2 in MDD and MDD treated with SSRI, respectively, User-defined: 0.05. The significance level for statistical tests was 0.05.

**Table 2**

Rs1800866 in *SIGMAR1* and major depressive disorder in the logistic regression analysis.

rs1800866	MDD, n (%)	Controls, n (%)	P-value <sup>a</sup>	OR <sup>b</sup>	95% CI <sup>c</sup>
AA	254 (54.5)	236 (45.7)			
AC	182 (39.1)	233 (45.2)	0.743	1.09	0.661–1.77
CC	30 (0.0644)	47 (9.11)	<b>0.0202</b>	0.563	0.346–0.913

Reference genotype is AA. Adjustment for age and gender.

<sup>a</sup> Bold numbers represent significant P-value.

<sup>b</sup> Odds ratio.

<sup>c</sup> 95% confidence interval.

3. Results

LD between rs1800866 and rs10814130 was very tight in our control samples ( $r^2$  more than 0.9) (Fig. 1). Therefore, we selected rs1800866 for the association analysis.

Genotype frequencies of rs1800866 were in HWE. We performed a logistic regression analysis to compare the phenotype among rs1800866 genotypes to adjust possible confounding. In the logistic regression analysis, we detected an association of the phenotypes (MDD or controls) with rs1800866 genotype (Table 2).

Among the clinical characteristics of the patients in this study, a significant difference with total SIGH-D score was detected at the baseline in relation to SSRI therapeutic response ( $P$ -value = 0.00140) (Table 1). We also found another significant difference in SSRI dose at eight weeks between remitters and nonremitters ( $P$ -value = 0.0462) (Table 1). In addition to SSRI treatment in this cohort, one patient each was prescribed alprazolam, loflazepate, and bromazepam. Three patients each were prescribed estazolam, etizolam and quazepam. Sixteen patients each were prescribed triazolam and flunitrazepam. Twenty-five patients each were prescribed zolpidem. In the logistic regression analysis, we detected the significant correlation between SIGH-D total score at the baseline and SSRIs the therapeutic response ( $P$ -value: 0.00346). Also, SSRIs dose at eight weeks also did not correlate with remission ( $P$ -value: 0.486). However, response or remission with SSRI treatment, were not associated with rs1800866 (Table 3).

Although we performed mutation screening in *SIGMAR1* using MDD treated by SSRIs samples, we detected three SNPs (rs10814130, rs1800866 and rs1799729), which are already known (Fig. 1). LD among rs10814130, rs1800866 and rs1799729 was very tight ( $r^2$  more than 0.9) (Fig. 1). LD from rs1799729 and rs1800866 in our controls was complete linkage disequilibrium, the same as in Uchida's study (Uchida et al., 2003). We did not find a novel SNP in *SIGMAR1*. Also, other previously reported SNPs were not detected in the Japanese MDD patients.

**Table 3**

Logistic regression analysis of rs1800866 in *SIGMAR1* in both definition groups.

rs1800866	Responder, n (%)	Nonresponder, n (%)	P-value <sup>a</sup>	OR <sup>b</sup>	95% CI <sup>c</sup>
AA	54 (46.2)	54 (59.3)			
AC	56 (47.9)	34 (37.4)	0.899	0.928	0.273–2.85
CC	7 (5.98)	3 (3.30)	0.273	2.88	0.476–23.9
rs1800866	Remitter, n (%)	Nonremitter, n (%)	P-value <sup>a</sup>	OR <sup>b</sup>	95% CI <sup>c</sup>
AA	39 (45.3)	69 (56.6)			
AC	40 (46.5)	50 (41.0)	0.484	0.667	0.199–2.00
CC	7 (8.14)	3 (2.46)	0.0769	5.49	0.915–45.4

Reference genotype is AA. Adjustment for age, gender, SIGH-D score at baseline, SSRI dose at 8 weeks and number of episode.

<sup>a</sup> Bold numbers represent significant P-value.

<sup>b</sup> Odds ratio.

<sup>c</sup> 95% confidence interval.

In the power analysis, we obtained more than 80% power for the detection of association when we set the genotype relative risk at 1.35 and 2.28 in MDD and SSRI response in MDD, respectively, for rs1800866 in *SIGMAR1* under a multiplicative model of inheritance.

#### 4. Discussion

We first performed a gene-based association analysis between *SIGMAR1* and MDD in the Japanese population. We performed an analysis of the possible correlations between phenotype and rs1800866 genotype and several clinical factors using logistic regression. In the logistic regression analysis, we detected an association of the phenotypes (MDD or controls) with rs1800866 genotype. Our results suggest that *SIGMAR1* plays a role in the pathophysiology of MDD in the Japanese population. On the other hand, the significant difference in baseline SIGH-D scores between responders and nonresponders might have affected the results; the baseline SIGH-D scores could predict clinical response in the logistic regression analysis. We did not find an association between rs1800866 genotype and SSRI therapeutic response in the logistic regression analysis. Remission with fluvoxamine or sertraline was marginally associated with rs1800866 in the case-control study. We also found another significant difference in SSRI dose at eight weeks between remitters and nonremitters. Therefore, we also performed an analysis of the possible correlations between remission with SSRI treatment and several clinical factors using logistic regression. Rs1800866 genotype was not predictive of the therapeutic remission. Therefore, we suggest that rs1800866 in the *SIGMAR1* genotype was not a predictor of SSRI treatment remission in Japanese MDD.

There was a significantly lower percentage of the C allele among MDD patients (MAF: 26.0%) than among controls (MAF: 31.7%). On the other hand, there was the trend toward higher percentage of the C allele among patients (MAF in responders and remitters: 29.9% and 31.5%, respectively) than among nonresponders and nonremitters (MAF in nonresponders and nonremitters: 22.0% and 23.0%, respectively). Rs1800866 (also known as A61C) was reported to be a possible functional SNP in exon 1, leading to an amino acid change from glutamine (CAG) to proline (CCG) at position 2 of the receptor protein (Seth et al., 1997). It is considered that this SNP may be a biologically functional SNP (Seth et al., 1997). LD from rs1800866 and rs1799729 in our MDD samples was completed as in Uchida's study using a Japanese sample (Uchida et al., 2003).

Miyatake and colleagues reported functional polymorphisms (T-485A) in the promoter region of *SIGMAR1* (Miyatake et al., 2004). However, MAF of this SNP is less than 5% according to studies of Miyatake and colleagues (Miyatake et al., 2004) and Satoh and colleagues (Satoh et al., 2004). Therefore, we designed the study based on the common disease-common variants hypothesis (CD–CV hypothesis) (Chakravarti, 1999), and did not perform an analysis on this SNP. Also, although we performed mutation screening in *SIGMAR1*, we did not detect this SNP. We considered that this result might have been caused by a small sample size.

Recently, Ishikawa and colleagues reported that rs1799729 and T-485A in *SIGMAR1* were not associated with fluvoxamine binding potential (Ishikawa et al., 2007). These authors suggested that rs1799729 and T-485A in *SIGMAR1* may not contribute to differences in sigma1 receptor in the human brain (Ishikawa et al., 2007). Moreover, they suggested that it will be necessary to conduct a replication study using larger samples, since T-485A, which is located in the promoter region and has a biologically functional effect, has very low MAF (less than 5% in the Japanese population) (Ishikawa et al., 2007).

Ishiguro and colleagues' haplotype analysis showed that SNPs in *SIGMAR1* (rs1799729 and rs1800866) were associated with schizophrenia (Ishiguro et al., 1998). These SNPs were also reported

to be associated with Japanese alcoholism (Miyatake et al., 2004) and schizophrenia (Ishiguro et al., 1998). In addition, rs1800866 was reported to be associated with Alzheimer disease/Alzheimer type dementia (Uchida et al., 2005). On the other hand, rs1799729 and rs1800866 in *SIGMAR1* were not associated with Japanese methamphetamine use disorder (Inada et al., 2004). Ohmori and colleagues reported that rs1799729 was not associated with Japanese schizophrenia (Ohmori et al., 2000), and Satoh and colleagues reported that SNPs in *SIGMAR1* (examined SNP: rs1799729, rs1800866 and T-485A) were not associated with Japanese schizophrenia (Satoh et al., 2004). In addition, Uchida and colleagues reported no association between rs1800866 and Japanese schizophrenia in a case-control study and a meta-analysis (Uchida et al., 2003).

A few points of caution should be mentioned with respect to our results. Firstly, the association between *SIGMAR1* and MDD patients may be due to biased samples, such as small sample sizes or unmatched age. We therefore performed a logistic regression analysis to compare the phenotypes among rs1800866 genotypes, using several clinical factors as other independent variables to adjust possible confounding. In the power analysis, we obtained more than 80% power for the detection of association when we set the genotype relative risk at 1.35 and 2.28 in MDD and SSRI response in MDD, respectively, for rs1800866 in *SIGMAR1* under a multiplicative model of inheritance. Because our samples were small, statistical errors are possible in the results of these statistical association analyses. On average, the controls were much younger than the patients. This means that a number of young controls may go on to develop one of these disorders, most likely MDD, since the incidence of major depression is as high as 5% or more. Our subjects did not undergo structured interviews, and it is reported that MDD patients who are not diagnosed by structured interview may develop bipolar disorder in the future (Bowden, 2001; Kishi et al., 2008, 2009a–d). However, in this study patients were carefully diagnosed according to DSM-IV criteria with consensus of at least two experienced psychiatrists on the basis of a review of medical records. In addition, when we found a patient who had been misdiagnosed, we promptly excluded the misdiagnosed case to maintain the precision of our sample (Kishi et al., 2008, 2009a–d). Also, Yamaguchi-Kabata and colleagues reported that different proportions of individuals from different regions of Japan in case and control groups can lead to statistical error (Yamaguchi-Kabata et al., 2008). Therefore, the positive association with MDD could be due to type I error, possibly because of population stratification. Secondly, we performed a mutation scan of *SIGMAR1*. Thirdly, the analysis of epigenetic controls and other genetic variations such as CNV in *SIGMAR1* were not performed in our study. Fourthly, sixty-nine of the 208 fluvoxamine and sertraline treatment patients were prescribed anxiolytics/hypnotic. Since benzodiazepines are also effective antidepressant molecules, our results for these 69 MDD patients may have been influenced by the anxiolytics/hypnotic. Because we consider it to be difficult to evaluate the association of extremely rare variants from the viewpoint of statistical power, a replication study using a larger sample will be required for conclusive results.

Our results suggest that *SIGMAR1* plays a role in MDD in the Japanese population. Also, *SIGMAR1* does not play a role in the therapeutic response to SSRI in Japanese MDD patients. However, because our samples are small, it will be important to replicate and confirm these findings in other independent studies using larger samples.

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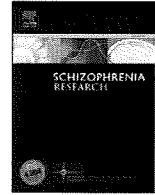
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## Genetic association study of KREMEN1 and DKK1 and schizophrenia in a Japanese population

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### ABSTRACT

The aim of the current study was to examine the association of KREMEN1 and DKK1, two wnt pathway-related genes with schizophrenia in Japanese subjects. We genotyped 16 common genetic variants within the aforementioned genes and examined their associations with schizophrenia. Results demonstrated that a common variant in the promoter region of KREMEN1 might modulate the risk of schizophrenia in the Japanese. However, further replication will be needed for conclusive interpretation of the effect of this locus on the pathogenesis of schizophrenia.

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### 1. Introduction

Irregularities consistent with abnormal brain development, including faulty neuronal migration, altered spatial neuronal arrangement, and absence of significant gliosis, have been reported in schizophrenia. Together with behavioral, neuro-motor, and other functional abnormalities that occur in childhood and predict schizophrenia, such as low IQ, poor

motor skills, and poor development of language and social skills, these morphological findings indicate a developmental origin for schizophrenia (Crow 1995, Benes et al., 1986).

Recent advances in the understanding of genes that regulate brain development offer insights into the mechanisms of developmental brain changes. One important issue is the identification of signaling pathways that coordinate changes in gene expression with dynamic changes in cell adhesion and migration, events that are important for the complex cellular architecture of the human brain. Although several growth factors affect both gene expression and cell migration, recent focus has been on the wnt signaling pathway (Clevers, 2006). wnts are powerful regulators of cell proliferation and differentiation, and their signaling pathway involves proteins that directly participate in both gene transcription and cell adhesion

**Abbreviations:** SNP, single nucleotide polymorphism; tSNP, tagging SNP; HWE, Hardy–Weinberg equilibrium; MDR, multifactor dimensionality reduction method; TA, testing accuracy; CVC, cross-validation consistency.

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(currently > 150 genes (Li et al., 2006). Because schizophrenia involves developmental brain changes and abnormal neuronal and synaptic organization, alterations in the transduction of wnt signaling pathway may represent such a mechanism (Clevers, 2006; Emamian et al., 2004). The genetic associations of several wnt pathway genes and schizophrenia have been reported (Proitsi et al., 2008). As coordinate changes in gene expression are important for normal brain development, the present study focused on negative modulators of the wnt signaling pathway, KREMEN1 and DKK1.

wnt/beta-catenin signaling is inhibited by the secreted protein Dickkopf1, a member of a multigene family, which induces head formation in amphibian embryos (Niehrs, 2006). Dickkopf1 inhibits wnt signaling by triggering LRP5/6 internalization through formation of a ternary complex with Kremen receptors (Mao et al., 2002). DKK1 encodes a protein that is a member of the dickkopf family. This gene maps on chromosome 10 region q11.2, a region with suggestive linkage evidence for schizophrenia (Faraone et al., 1998). KREMEN1 encodes a high-affinity dickkopf homolog 1, a transmembrane receptor that functionally cooperates with dickkopf1 to block wnt/beta-catenin signaling. KREMEN1 maps to chromosome 22q12.1, relatively close to the region with weak, but suggestive linkage evidence for schizophrenia (Pulver et al., 1994).

We genotyped 16 common genetic variants within the aforementioned genes and examined their associations with schizophrenia. Additionally, we examined interactions between single nucleotide polymorphisms (SNPs) within KREMEN1 and DKK1.

## 2. Materials and methods

Subjects comprised 1624 patients with schizophrenia (mean age,  $46.5 \pm 14.5$  years) and 1621 volunteers with no personal or family history of psychiatric illness (mean age,  $45.1 \pm 14.0$  years). Subjects were divided into a screening sample ( $n = 1681$ ) and a confirmation sample ( $n = 1564$ ). A general characterization and psychiatric assessment of subjects is available elsewhere (Ikeda et al., 2008). The screening and confirmation sample were collected independently at each university hospital. All subjects provided written informed consent. This study was approved by the ethics committee at Nagoya University.

DNA was extracted from peripheral blood. Genotyping was performed using a fluorescence-based allelic discrimination assay (Taqman, Applied Biosystems, Foster City, CA, USA). Power was calculated according to the methods of Skol et al (Skol et al., 2006).

SNP tagging criteria were based on minor allele frequency (>5%) and correlation coefficient between loci (>0.8) as reported in the HAPMAP database (rel #24, Japanese population). Based on these criteria, 16 tagging SNPs (tSNP) were selected for genotyping. To exclude low-quality DNA sample or genotyping probes, data sets were filtered on the basis of tSNP genotype call rates (95% completeness) or deviation from the Hardy-Weinberg equilibrium (HWE) ( $P = 0.05$ ) in the control sample. Subjects whose percentage of missing genotypes was >10% or who had evidence of possible DNA contamination were excluded from subsequent analyses.

All allele-wise association analyses (screening or confirmation sample) were carried out by calculating the  $P$  values for each tSNP. Significance was determined at the 0.05 level

using Fisher's exact test. All  $P$  values were two-sided. Log likelihood ratio tests for assessing haplotype-wise associations between schizophrenia and combination of tagging SNPs was performed using UNPHASED software v3.04 (Dudbridge, 2008). The rare haplotype frequency threshold was set at 5%.

To reduce the total number of tests, clearly unassociated markers were removed in the first stage involving the screening sample of the present study. Next, conditional on the findings of the first stage, which used a less stringent nominal level, was tested in the second stage involving the confirmation sample using the augmented data and data from the first stage. In this joint sample analysis,  $P$  values were generated by Cochran-Mantel-Haenszel stratified analysis, and the Breslow-Day test was performed for evaluation of heterogeneous associations as implemented in PLINK v1.06 (Purcell et al., 2007).

To determine high- and low-risk genotype combinations, the multifactor dimensionality reduction method (MDR) was used (Moore et al., 2006). This procedure was developed to detect higher-order interactions between polymorphisms to predict a dichotomous trait variation, even when marginal effects are small. A 10-fold cross-validation test was conducted. Accordingly, a model was constructed based on 9/10 of the data (training data) and then evaluated using the remaining 1/10 of the data (testing data). The fitness of the MDR model was assessed by estimating the testing accuracy (TA). Models that were true positive would have an estimated TA of >0.5. The cross-validation consistency (CVC) was a measure of how many times among 10 divisions of the data the MDR found the same best model. We used the Tuned Relief algorithm (Moore and White, 2007) to removed noisy SNPs from the pool of possible candidates. Statistical significance was evaluated using a 1000-fold permutation test to compare observed testing accuracies with those expected under the null hypothesis. Permutation testing corrects for multiple testing by repeating the entire analysis on 1,000 data sets that are consistent with the null hypothesis. Models were significant at the 0.01 level.

## 3. Results

The results of the power analysis are presented in Table 1. We genotyped 16 tSNPs using the screening sample. All tSNPs were in HWE, and the SNP-wise call rate was >95%. The association signal was detected in allele-wise analysis at rs713526 as well as in the haplotype-wise analysis (Table 2). To deal with multiple testing issues and to keep the statistical power uncompromised, we performed additional genotyping of rs713526 using the confirmation sample. Although the direction of the association was the same, no statistically significant results were obtained (Table 3). To maximize statistical power, we performed joint association analysis combining the data from the screening and confirmation sample and obtained a positive association signal.

We performed an exploratory SNP-SNP interaction analysis. For a specific number of SNPs we reported a single best model that maximized the TA. This is the model that is most likely to generalize to independent data sets. We also reported the CVC, which represents the number of times in a particular cross validated run that a given SNP combination was selected as the best model. The strongest evidence for SNP interaction was between rs1896368 (DKK1), rs2288335 (DKK1), and rs5752866

**Table 1**  
Power analysis.

		Screening sample		Confirmation sample		Joint analysis	
		$P=0.05^a$	$P=0.001^a$	$P=0.05^a$	$P=0.001^a$	$P=0.05^a$	$P=0.001^a$
GRR <sup>b</sup>	0.05 <sup>c</sup>	1.51	1.78	1.50	1.76	1.30	1.56
	0.30 <sup>c</sup>	1.25	1.36	1.24	1.35	1.18	1.26
Number of SNPs		16		1		1	
Sample size	Cases	831		793		1624	
	Controls	850		771		1621	
Total		1681		1564		3245	

<sup>a</sup>  $P$ -value.<sup>b</sup> Genotype relative risk in order to achieve 80% of power at given minor allele frequency (prevalence = 0.01).<sup>c</sup> Minor allele frequencies.

(KREMEN1), which predicted disease status correctly 55.76% of the time (Table 4).

#### 4. Discussion

We conducted this study on two genes related to the wnt signaling pathway. The study is relatively large (>3000 case-control subjects), and a pathway-based approach was used for candidate gene selection. Results of this study provide genetic evidence for the involvement of KREMEN1 locus in schizo-

phrenia. The associated SNP is located in the promoter region of KREMEN1. This association was also observed in haplotype-wise analysis. The observed association at KREMEN1 locus was stronger in the single marker analysis (compared with haplotype analysis), which might be an indication that rs713526 is a better proxy of genetic association than the associated haplotype. Results from the screening sample could not be replicated in the confirmation sample (probably due to relatively low power), but combined analysis yielded evidence for a genetic association.

**Table 2**  
Results (Screening sample).

Gene	dbSNP	Minor allele	Screening sample							
			Single marker (allelewise)			Multimarker (sliding window) <sup>a</sup>				
			Cases <sup>b</sup>	Controls <sup>b</sup>	$P^c$	195 <sup>d</sup>	U95 <sup>d</sup>	2 markers	3 markers	4 markers
DKK1	rs1896368	T	0.407	0.397	0.586	0.903	1.198	0.863		
	rs1896367	T	0.288	0.289	0.954	0.855	1.159	0.874	0.943	0.620
	rs1528877	G	0.288	0.293	0.744	0.837	1.135	0.254	0.414	0.626
	rs1569198	G	0.199	0.219	0.164	0.748	1.051	0.383	0.435	
	rs2288335	A	0.116	0.115	0.875	0.820	1.263			
KREMEN1	rs134603	T	0.307	0.315	0.646	0.831	1.122	0.117		
	rs134656	C	0.332	0.342	0.541	0.825	1.106	0.585	0.764	0.824
	rs5752866	A	0.475	0.453	0.220	0.949	1.253	0.585	0.673	0.785
	rs134672	T	0.176	0.180	0.750	0.810	1.164	0.925	0.681	0.730
	rs8135301	T	0.108	0.112	0.731	0.771	1.200	0.768	0.858	0.787
	rs134683	A	0.234	0.245	0.452	0.799	1.105	0.603	0.678	0.575
	rs5762996	A	0.467	0.451	0.362	0.928	1.226	0.575	0.857	0.764
	rs132277	T	0.309	0.328	0.254	0.791	1.064	0.371	0.596	0.211
	rs5763001	A	0.130	0.119	0.315	0.903	1.374	0.380	0.219	0.236
	rs2301446	A	0.107	0.098	0.429	0.872	1.379	0.026	0.070	
	rs713526	A	0.152	0.186	0.009	0.650	0.941			

<sup>a</sup> Log likelihood ratio test  $P$  value.<sup>b</sup> Minor allele frequency.<sup>c</sup> Fisher's exact test.<sup>d</sup> 95% confidence intervals (odds ratio).

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**Table 3**  
Results (confirmation sample).

Gene	dbSNP	Minor allele	Confirmation sample					Joint analysis					
			Single marker (allelewise)					Single marker (allelewise)					
			Cases <sup>a</sup>	Controls <sup>a</sup>	P <sup>b</sup>	L95 <sup>c</sup>	U95 <sup>c</sup>	Cases <sup>a</sup>	Controls	P <sup>d</sup>	L95 <sup>c</sup>	U95 <sup>c</sup>	P <sub>Bd</sub> <sup>e</sup>
KREMEN1	rs713526	A	0.155	0.172	0.193	0.726	1.067	0.155	0.177	0.018	0.745	0.9727	0.642

<sup>a</sup> Minor allele frequency.<sup>b</sup> Fisher's exact test.<sup>c</sup> 95% confidence intervals (odds ratio).<sup>d</sup> Cochran–Mantel–Haenszel test.<sup>e</sup> Breslow–Day test.**Table 4**  
SNP–SNP interaction.

Model <sup>a</sup>				TA <sup>b</sup>	CVC <sup>c</sup>	P value <sup>d</sup>
rs1896368 (DKK1)	rs2288335 (DKK1)	rs5752866 (KREMEN1)		0.5576	10	<0.001
rs1528877 (DKK1)	rs1896368 (DKK1)	rs8135301 (KREMEN1)	rs5752866 (KREMEN1)	0.5542	10	<0.01
rs1528877 (DKK1)	rs1896368 (DKK1)			0.5484	7	<0.01

<sup>a</sup> Best model for particular number of SNPs.<sup>b</sup> Testing accuracy.<sup>c</sup> Cross validation consistency (out of 10).<sup>d</sup> Based on 1000 permutations.

After examining the main effects of each SNP, we analyzed potential interactions between these SNPs. It was of interest to identify interactions that predict the risk for schizophrenia, even in the absence of main effects. The tSNPs kept in the optimal MDR model were not associated with schizophrenia in single-locus association analysis, which is consistent with epistasis, as the effect of one locus may be too weak to be detected when the effect of another locus is not accounted for.

Some limitations are inherent in this type of study. Our study is not comprehensive, as we did not conduct a study regarding all wnt signaling pathway genes in the linkage regions for schizophrenia. Another potential concern was population admixture, a known confounding factor for association. The Japanese population has a small genetic diversity (Haga et al., 2002). However, even in such a genetically homogeneous population, a small amount of stratification may produce a spurious genetic association signal (Yamaguchi-Kabata et al., 2008).

Our study demonstrated that a representative tSNP of KREMEN1 might modulate the risk of schizophrenia in the Japanese population. However, these results were obtained solely through the genetic analysis, and pathophysiological explanations need to be determined. We conclude that these results are suggestive of an association with schizophrenia at this locus, but that our findings cannot be considered conclusive without replication.

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#### Contributors

BA, IK, YI and YN performed laboratory assays and the data-analysis. BA drafted the manuscript. HU, MS, TI, RH, MT, NI and NO participated in the design of the study, and coordinated sample collection. All authors contributed to and have approved the final manuscript.

#### Conflict of interest

The authors have no financial conflicts to declare.

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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.<sup>1–3</sup> Genetic studies also have implicated the ubiquitin–proteasome system in a range of neuropsychiatric diseases, such as Parkinson's disease,<sup>4</sup> autism spectrum disorders,<sup>5,6</sup> mental retardation,<sup>7–9</sup> bipolar disorder<sup>10,11</sup> and schizophrenia.<sup>11,12</sup>

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.<sup>13</sup> *Usp46* is one of approximately a hundred deubiquitinating enzymes. Protein deubiquitination by deubiquitinating enzymes can either antagonize or facilitate substrate presentation to the proteasome.<sup>2</sup> Deubiquitinating enzymes have also been associated with neurogenetic disorders, including Parkinson's disease,

spinocerebellar ataxia. In the aforementioned study,<sup>13</sup> 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,<sup>14</sup> were also reported in the mice.<sup>13</sup> Furthermore, the *USP46* locus (4q12) corresponds to the linkage regions for bipolar disorder.<sup>15</sup> 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<sup>16</sup> and a population-based epidemiological study<sup>17</sup> 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<sup>18</sup> 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.<sup>19</sup> 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.<sup>20</sup> 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  $\chi^2$ -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  $\chi^2$ -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/>).<sup>21</sup> 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<sup>22</sup> 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**

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

Abbreviations: M, major allele; m, minor allele; SNP, single-nucleotide polymorphism.

<sup>a</sup>Sliding window analysis, rare haplotype threshold 10%.**Table 3 Allele-/genotype-/haplotype-wise analyses in schizophrenia**

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

Abbreviations: M, major allele; m, minor allele; SNP, single-nucleotide polymorphism.

<sup>a</sup>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.<sup>23</sup> 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.*<sup>24</sup>, *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.<sup>25</sup> 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<sup>26</sup> in search