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QTc prolongation and antipsychotic medications in a sample of 1017 patients with schizophrenia

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ABSTRACT

Many antipsychotic drugs cause QT prolongation, although the effect differs based on the particular drug. We sought to determine the potential for antipsychotic drugs to prolong the QTc interval (>470 ms in men and >480 ms in women) using the Bazett formula in a "real-world" setting by analyzing the electrocardiograms of 1017 patients suffering from schizophrenia. Using logistic regression analysis to calculate the adjusted relative risk (RR), we found that chlorpromazine (RR for 100 mg = 1.37, 95% confidence interval (CI) = 1.14 to 1.64; $p<.005$), intravenous haloperidol (RR for 2 mg = 1.29, 95% CI = 1.18 to 1.43; $p<.001$), and sultopride (RR for 200 mg = 1.45, 95% CI = 1.28 to 1.63; $p<.001$) were associated with an increased risk of QTc prolongation. Levomepromazine also significantly lengthened the QTc interval. The second-generation antipsychotic drugs (i.e., olanzapine, quetiapine, risperidone, and zotepine), mood stabilizers, benzodiazepines, and antiparkinsonian drugs did not prolong the QTc interval. Our results suggest that second-generation antipsychotic drugs are generally less likely than first-generation antipsychotic drugs to produce QTc interval prolongation, which may be of use in clinical decision making concerning the choice of antipsychotic medication.

1. Introduction

QTc interval prolongation is associated with presyncope, syncope, polymorphic ventricular tachycardia, the subtype torsade de pointes, and sudden cardiac death (Faber et al., 1994). Previous studies have indicated an increased risk of sudden cardiac death in patients treated with antipsychotics (Hennessy et al., 2002; Ray et al., 2001; Straus et al., 2004). A retrospective cohort study of 481,744 Tennessee Medicaid enrollees, of whom 1487 died from sudden cardiac death, found that current moderate-dose antipsychotic use (>100 mg of thioridazine equivalents) increased the rate of sudden cardiac death (multivariate risk ratio of 2.39), when compared with the nonuse of antipsychotics (Ray et al., 2001). A cohort study of three U.S. medical programs found that patients with treated schizophrenia had higher rates of cardiac arrest and ventricular arrhythmia than did controls (patients with glaucoma and those with psoriasis), with risk ratios ranging from 1.7 to 3.2 (Hennessy et al., 2002). A study of 554 sudden cardiac death subjects reported that the current use of antipsychotics was associated with a three-fold increased risk of cardiac death (Straus et al., 2004).

Although torsade de pointes and sudden death are rare, rate-corrected QT (QTc) prolongation serves as a risk factor for these conditions. In a study of 495 psychiatric patients receiving various psychotropic drugs and 101 healthy reference individuals, 8% of patients showed QTc prolongation (>456 ms) (Reilly et al., 2000). Advanced age (>65 years), as well as the use of tricyclic antidepressants, thioridazine, and droperidol were indicated as robust predictors of QTc lengthening (Reilly et al., 2000). High antipsychotic doses were also associated with QTc prolongation (Reilly et al., 2000). In a sample of 111 psychiatric inpatients receiving a median daily dose of more than 600 mg [chlorpromazine (CP) equivalent] of antipsychotics, 90% had schizophrenia or related psychoses, and 23% showed QTc interval of >420 ms, whereas only 2% of unmedicated controls did (Warner et al., 1996). However, there is little clinical data to aid in assessing the

Abbreviations: QTc, rate-corrected QT; 95% CI, 95% confidence interval; HPD, haloperidol; HPDiv, intravenous injection of haloperidol; RR, relative risk; ECG, electrocardiogram; SGAs, second-generation antipsychotics; FGAs, first-generation antipsychotics; DSM-IV, Diagnostic and Statistical Manual of Mental Disorders, 4th ed.; CP, chlorpromazine; LP, levomepromazine; OR, odds ratio.

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risk of QTc prolongation for an individual antipsychotic in a dose-dependent manner, particularly for second-generation antipsychotics (SGAs). Some case reports have indicated that SGAs can induce QTc prolongation (Dineen et al., 2003; Vieweg, 2003). However, such anecdotal reports do not provide clear evidence of whether SGAs increase the risk of QTc prolongation, as in first-generation antipsychotics (FGAs), in a real-world setting. This study examined the risk of QTc prolongation of antipsychotic drugs in a large clinical sample from Japan. Japan is known to use higher doses of antipsychotics (Bitter et al., 2003), providing a unique opportunity to investigate the risk of QTc prolongation in a wide range of antipsychotic doses.

2. Methods

2.1. Patients

Clinical information, including data on QTc intervals, was collected from inpatients with schizophrenia who were diagnosed according to the Diagnostic and Statistical Manual of Mental Disorders, 4th ed. (DSM-IV) in four independent hospitals. Approval from the ethics committee of each hospital was obtained. Data collection on all inpatients with schizophrenia was begun on the following dates in three psychiatric hospitals Biwako Hospital, Toyosato Hospital, and Minakuchi Hospital: February 2, 2007; February 3, 2007; and July 29, 2007, respectively. In the fourth hospital, the National Center of Neurology and Psychiatry Hospital, clinical records were collected for all patients who were admitted to its psychiatric wards between 1998 and 2007. A total of 1065 inpatients were included from the four hospitals, and all of them underwent ECG screening. Among them, 37 patients were excluded due to hypokalemia (serum potassium <3.5 mEq/L), which can induce QTc interval prolongation (Elming et al., 2003; Taylor, 2003). Two were excluded because of hypothyroidism, and nine because of cardiac disease (four patients with right bundle branch block, two with post-acute myocardial infarction, one with WPW syndrome, one with atrial–ventricular block, and one who underwent surgery for atrial septal defect). The remaining 1017 patients had a mean age of 42.6 years (S.D., 18.2) and were included in the analysis.

2.2. Procedure

A standard 12-lead ECG was recorded at 25 mm/s. Because the QTc interval is influenced by heart rate, it was corrected by Bazett's formula (QTc: $QTc = QT/RR^{1/2}$) (Bazett, 1920). An ECG recording showing the longest QTc interval was selected for each patient whose ECG was recorded two or more times. The QTc was measured automatically by a program on the ECG apparatus (MAC 5500 with 12SL algorithm by GE health care [Amersham Place, Little Chalfont, Buckinghamshire, UK]). For patients with a QTc > 430 ms, QTc and RR intervals were measured manually for the chest lead with the maximal T-wave amplitude, according to Charbit et al. (2006). The end of the T-wave was determined as the intersection between the tangent to the steepest downslope of the T-wave and the isoelectric line. QTc prolongation was defined as a QTc length of more than 470 ms in males and more than 480 ms in females, as 99% of "healthy" people can be excluded by this cut-off value (Taggart et al., 2007). One of the coauthors (M.H.), a cardiologist who specializes in arrhythmias, trained the authors on how to evaluate an ECG recording. Information on drugs administered within 24 h of the ECG recording was obtained. Table 1 shows the distribution of drugs that were administered in more than 3% of the patients and the prevalence of QTc prolongation for each medication. One hundred forty-two patients were drug free when the ECG was recorded, because they were given the test at admission before they had taken any drugs. Two hundred sixty-five patients were on monotherapy. Doses of antipsychotics, antiparkinsonian drugs, and benzodiazepines were converted into those of CP, biperiden, and diazepam equivalents, respectively (Inagaki and Inada, 2006). Subjects who were coadministered medical drugs (i.e., non psychotropic drugs) with an increased risk of producing torsade de pointes were excluded (Chan et al., 2007).

Table 1
Medication and rate of QTc prolongation in 1017 patients. Drugs which were administrated to more than 3% of patients are shown.

Administrated drugs	No. of Patients $n = 1017$ (100%)	Mean dose (SD), mg	No. of patients (%) with QTc prolongation (male: >470 ms, female >480 ms)
Equivalent dose			
CP eq.	875 (86)	963.0 (879.0)	23 (2.6)
Diazepam eq.	672 (66)	14.6 (14.6)	18 (2.7)
Biperiden eq.	645 (63)	3.8 (2.2)	19 (2.9)
Mood stabilizer			
CBZ	74 (7)	478.9 (201.8)	3 (4.1)
VPA	54 (5)	650.0 (334.1)	1 (1.9)
Lithium	47 (5)	587.2 (199.6)	4 (8.5)
Antipsychotics			
HPD	375 (37)	15.9 (12.6)	16 (4.3)
CP	299 (29)	190.5 (198.7)	9 (3.0)
LP	258 (25)	91.9 (94.5)	14 (5.4)
Risperidone	248 (24)	5.6 (3.7)	4 (1.6)
Zotepine	116 (11)	179.9 (124.9)	3 (2.6)
Olanzapine	104 (10)	15.6 (6.4)	0 (0.0)
Quetiapine	60 (6)	375.5 (258.5)	0 (0.0)
Bromperidol	49 (5)	10.7 (8.6)	0 (0.0)
Sultopride	49 (5)	1032.9 (810.2)	10 (20.4)
HPD iv	47 (5)	16.0 (10.5)	8 (17.0)

Abbreviations: eq = equivalent; HPD = haloperidol, CP = chlorpromazine; LP = levomepromazine, CBZ = carbamazepine, VPA = sodium valproate; No. = Number, SD = standard deviation.

2.3. Statistical analyses

First, logistic regression analysis was applied to examine risk factors for QTc prolongation. Age, sex, antipsychotic dose (CP equivalent), benzodiazepine dose (diazepam equivalent), and antiparkinsonian drug dose (biperiden equivalent) were included in the backward stepwise regression model. In the second analysis, age, sex, and individual antipsychotic doses were entered as independent variables in the logistic regression analysis. Then, the adjusted relative risks of important explanatory variables were calculated via the backward stepwise regression analysis. Drugs that were administrated in more than 3% of the patients were analyzed.

Linear regression analysis was used to determine which antipsychotics lengthened the QTc interval in a dose-dependent manner, as the antipsychotic dose was entered as a continuous variable. Then, the adjusted coefficients were calculated using the stepwise selection model. Age, sex, and individual antipsychotic doses were entered as independent variables.

The χ^2 test was used to examine the risk-increasing effect of excessive use of antipsychotics (cut-off points of 1000 or 1500 mg/day of CP equivalent). All statistical analyses were performed using the SPSS, version 13.0 (SPSS Japan, Inc., Tokyo, Japan). All p-values reported are two tailed. Statistical significance was considered when p-value was less than 0.05.

3. Results

The prevalence of QTc prolongation (>470 ms in male and >480 ms in female) was 2.5% (male: 3.7%; female: 1.0%). Logistic regression analysis showed that the antipsychotic dose was a significant risk factor for QTc prolongation (Table 2), whereas antiparkinsonian drugs, benzodiazepines, and mood stabilizers were not risk factors for QTc prolongation. Administration of antipsychotic doses greater than 1000 and 1500 mg/day of CP equivalent was found

Table 2
Result of logistic regression analysis on the risk of QTc prolongation for standardized doses.

	Unadjusted relative risk (95% CI)	Adjusted relative risk (95% CI)
Age	0.97 (0.94–0.99)	
Sex (risk of female)	0.33 (0.12–0.95)	
CP eq. (100 mg)	1.08 (1.05–1.12)*	1.07 (1.04–1.10)*
Diazepam eq. (1 mg)	1.01 (0.98–1.04)	
Biperiden eq. (1 mg)	0.87 (0.72–1.06)	
CBZ (100 mg)	1.00 (1.00–1.00)	
VPA (100 mg)	1.00 (0.99–1.00)	
Lithium (100 mg)	1.00 (1.00–1.01)	
	The Hosmer–Lemeshow Goodness-of-Fit Test $x^2=4.77$ df=8 $p=0.85$	The Hosmer–Lemeshow Goodness-of-Fit Test $x^2=5.15$ df=8 $p=0.74$

*$p<0.001$.
Abbreviations: eq = equivalent, CP = chlorpromazine, CBZ = carbamazepine; VPA = sodium valproate, CI = confidence interval.

to increase the risk of QTc prolongation 1.97 fold (95% CI, 1.48–2.59, $p<0.001$) and 2.76 fold (95% CI, 1.80–4.18, $p<0.001$), respectively, when compared to their counterparts. On examination of individual antipsychotics, haloperidol intravenous injection (HPDiv), CP, and sultopride were found to increase the risk of QTc prolongation (Table 3).

In the stepwise selection model of the multiple linear regression analysis, CP, HPDiv, levomepromazine (LP), and sultopride were found to lengthen the QTc interval. Age was also indicated as a risk factor for QTc lengthening. Adjusted coefficients for CP, HPDiv, LP, sultopride, and sex are shown in Table 4. Adding 100 mg of LP, for example, extended the QTc interval by 4.65 ms. Bromperidol, olanzapine, quetiapine, risperidone, and zotepine had no significant lengthening effect on the QTc interval.

Table 3
Result of logistic regression analysis on the risk of QTc prolongation for each antipsychotic drug.

	Unadjusted relative risk (95%CI)	Adjusted relative risk (95%CI)
Age	0.99 (0.96–1.03)	
Sex (risk of female)	0.38 (1.26–1.16)	
HPD (2 mg)	0.99 (0.92–1.06)	
CP (100 mg)	1.37 (1.13–1.67)*	1.37 (1.14–1.64)*
LP (100 mg)	1.55 (0.92–2.61)	
Risperidone (1 mg)	1.01 (0.84–1.12)	
Zotepine (66 mg)	0.91 (0.62–1.34)	
Olanzapine (2.5 mg)	0.00 (0.00 to >100)	
Quetiapine (66 mg)	0.00 (0.00 to >100)	
Bromperidol (2 mg)	0.00 (0.00 to >100)	
Sultopride (200 mg)	1.40 (1.23–1.60)**	1.45 (1.28–1.63)**
HPD iv (2 mg)	1.26 (1.13–1.40)**	1.29 (1.18–1.43)**
	The Hosmer–Lemeshow Goodness-of-Fit Test $x^2=5.04$ df=8 $p=0.75$	The Hosmer–Lemeshow Goodness-of-Fit Test $x^2=17.81$ df=8 $p=0.013$

*$p<0.005$.
**$p<0.001$.
Abbreviations: HPD = haloperidol, CP = chlorpromazine, LP = levomepromazine, iv = intravenous injection, CI = confidence interval.

4. Discussion

In a large clinical sample, we confirmed that a daily dose of antipsychotics (CP equivalents) was associated with a dose-dependent increased risk of QTc prolongation; however, the use of antiparkinsonian drugs, benzodiazepines, and mood stabilizers did not significantly increase this risk. With regard to individual antipsychotics, CP, HPDiv, and sultopride were shown to significantly increase the risk of QTc prolongation. CP, HPDiv, LP, and sultopride were found to significantly lengthen the QTc interval, whereas HPD, bromperidol, olanzapine, quetiapine, risperidone, and zotepine were not.

Our observation that a daily dose of antipsychotics was associated with a risk of QTc prolongation is consistent with previous studies (Reilly et al., 2000; Warner et al., 1996). In our sample, antipsychotic doses of more than 1000 and 1500 mg/day of CP equivalents were found to increase the risk of QTc prolongation by approximately 2.0 and 3.0 fold, respectively, when compared to their counterparts. Reilly et al. also reported that a high dose (1000 to 2000 mg/day) and a very high dose (>2000 mg/day) predicted QTc prolongation [odds ratio (OR), 5.3 and 8.2, respectively] (Reilly et al., 2000). Warner et al. reported an OR of 4.3 for doses higher than 2000 mg/day (Warner et al., 1996). In contrast to antipsychotics, mood stabilizers showed no significant risk-increasing effect. This is consistent with a previous finding, which showed that lithium or carbamazepine did not significantly increase the risk of QTc prolongation (Reilly et al., 2000). However, a recent study suggested that lithium increases the QTc interval significantly (18.6 ms; 95% CI, 4.8–32.4 ms) (van Noord et al., 2009). Furthermore, lithium is known to cause T-wave changes (Mitchell and Mackenzie, 1982; Reilly et al., 2000) that may lead to torsade de pointes when combined with a QTc-lengthening antipsychotic (Liberatore and Robinson, 1984). Thus, the use of lithium requires careful ECG monitoring. With respect to valproate, our study may be the first to investigate the risk of QTc prolongation for this drug in a clinical setting. With regard to coadministered benzodiazepine and antiparkinsonian drugs, our results suggest no significant effect on QTc prolongation. Although some patients taking diazepam and biperiden equivalent showed QTc interval prolongation (Table 1), the results of logistic regression analysis showed no significant risk-increasing effect of these drugs (Table 2). Therefore, these patients were also taking chlorpromazine equivalent and it was the chlorpromazine equivalent that explained the QTc interval prolongation. Indeed, to our knowledge, there has been no study reporting that these drugs cause QTc prolongation or torsade de pointes.

With respect to individual antipsychotics, previous studies have reported that thioridazine, intravenous droperidol, sertindole, and ziprasidone are associated with a strong risk-increasing effect on QTc prolongation (Czekalla et al., 2001a; Harrigan et al., 2004; Taylor,

Table 4
QTc prolongation effect of each antipsychotic by linear regression model.

	Forced entry model	Stepwise selection model
	Coefficient (95% CI)	Coefficient (95% CI)
Age	0.19 (0.10–0.28)*	0.20 (0.11–0.29)*
Sex (risk of female)	3.22 (−0.01–6.44)	
HPD (2 mg)	0.42 (0.09–0.76)	
CP (100 mg)	3.91 (2.69–5.13)*	3.82 (2.62–5.02)*
LP (100 mg)	4.87 (2.14–7.60)*	4.65 (1.94–7.37)*
Risperidone (1 mg)	0.07 (−0.47–0.61)	
Zotepine (66 mg)	−0.36 (−1.91–1.20)	
Olanzapine (2.5 mg)	0.30 (−0.47–1.08)	
Quetiapine (66 mg)	0.11 (−0.87–1.09)	
Bromperidol (2 mg)	0.08 (−1.00–1.16)	
Sultopride (200 mg)	3.65 (2.48–4.82)*	3.56 (2.41–4.72)*
HPD iv (2 mg)	3.16 (2.36–3.96)*	3.13 (2.34–3.93)*

*$p<0.001$.
Abbreviations: HPD = haloperidol, CP = chlorpromazine, LP = levomepromazine; iv = intravenous injection, CI = confidence interval.

2003). In Japan, commercial use of thioridazine ended in 2005; intravenous droperidol has not been used in psychiatric treatment; and sertindole and ziprasidone have not been introduced. Thus, we could not confirm the effect of these drugs. However, our results provide robust evidence that HPDiv increases the risk of QTc prolongation. This concurs with Hatta et al. who compared the differences in QTc length among psychiatric emergency patients who received intravenous flunitrazepam alone and those who received intravenous flunitrazepam and haloperidol and found that the latter group showed significantly longer QTc intervals than the former (Hatta et al., 2001). Vieweg et al. (2009) reviewed the literature and identified cases of patients aged ≥60 years who developed QTc interval prolongation, polymorphic ventricular tachycardia/torsade de pointes and/or sudden cardiac death while taking antipsychotic or antidepressant drugs or a combination of these medications. Among such cases, most frequently reported medication was HPDiv (14 out of 37 cases). These findings and ours support the recent alert of the U.S. Food and Drug Administration warning that HPDiv increases the risk of QTc prolongation and torsade de pointes based on at least 28 cases reported in the literature (U.S. Food and Drug Administration Cfdear, 2007). Oral HPD, in contrast, was found to have no statistically significant risk-increasing effect on QTc prolongation, although it had a significant QTc-lengthening effect. Previous findings have suggested that oral HPD at low or moderate doses had no clear effect on QTc, but that it is associated with QTc prolongation and torsade de pointes at higher clinical doses (>20 mg/day) (Czekalla et al., 2001a; Taylor, 2003). Taken together, excessively high blood levels of the drug after an intravenous injection or oral intake of high doses may be critical for the effect of HPD. Regarding bromperidol (oral use only), a chemically similar butyrophenone to HPD, we obtained no evidence for its effect on QTc prolongation or lengthening. To our knowledge, this is the first study to examine bromperidol for such effects. Further studies are warranted to confirm our results. With respect to CP, we detected significant effects on both QTc prolongation and QTc lengthening, which is consistent with previous findings, suggesting an intermediate effect of CP on QTc (i.e., a weaker effect than that of thioridazine, but stronger than oral HPD) (Czekalla et al., 2001a; Mehtonen et al., 1991; Witchel et al., 2003), although there have been some reports of no significant risk-increasing effect of CP (Reilly et al., 2000; Strachan et al., 2004). LP, another phenothiazine, was also found to lengthen the QTc interval in the multiple regression analysis. In the logistic regression, statistical significance was nearly achieved ($p=0.06$, Table 3). These results suggest that LP is likely to increase the risk of QTc prolongation. Although there have been little data on LP in relation to QTc in the literature, an association between sudden death and the use of phenothiazines is prominent, and LP might have been involved in such deaths (Mehtonen et al., 1991). Finally, sultopride, a benzamide derivative, was found to significantly increase the risk of QTc prolongation and QTc lengthening. To our knowledge, this is the first time that such evidence has been obtained for sultopride. Further studies are warranted to confirm our results.

Our results provide no evidence for the possible risk-increasing effect of the examined SGAs (olanzapine, quetiapine, risperidone, and zotepine) on QTc prolongation. Recently, Ray et al. (2009) reported that atypical antipsychotics double the risk of sudden cardiac death when compared with nonusers of antipsychotic drugs, a finding that contradicts our data. However, SGAs can induce weight gain, insulin resistance, and dyslipidemia (Tschoner et al., 2009), all of which are risk factors for ischemic heart diseases. Therefore, the increased sudden death observed by Ray et al. (2009) could be attributable to the increased risk of ischemic heart diseases rather than torsade de pointes due to QTc prolongation. The Pfizer 054 study (2000) reported that SGAs, such as risperidone, quetiapine, ziprasidone, and olanzapine, induced QTc interval prolongation. In the review of Czekalla et al. (2001a), it was suggested that risperidone and quetiapine could lengthen the QTc interval, although the effect observed was smaller than that of thioridazine and chlorpromazine. Olanzapine, in particular, was reported to have little effect on the QTc-interval length (Czekalla et al., 2001b). Dineen et al. (2003) reported the case of a patient who was treated with olanzapine and showed an abnormal QTc interval. Vieweg (2003) reviewed the literature and found nine cases in which QTc prolongation was associated with SGA administration (four cases of risperidone [one case was his original case], three cases of quetiapine, and two cases of ziprasidone). Taken together, although our results suggest that the SGAs (olanzapine, quetiapine, risperidone, and zotepine) are less likely to produce QTc interval prolongation than the FGAs examined herein, the SGAs can also cause QTc prolongation. Thus, further investigations with a more refined methodology are warranted. In particular, the current group-derived formula for correcting QT interval measurements to a heart rate of 60 beats per/min (QTc) are unsatisfactory (Malik, 2001), and, as pointed out by Vieweg (2003), determining the effect of drug-induced change amid the noise of random variation (regression to the mean) will require a new technology.

Female gender is known to be a risk factor for QTc prolongation (Taylor, 2003; Vieweg et al., 2009). However, we failed to detect female gender as a significant risk factor in our sample. Moreover, QTc prolongation was found more commonly in male patients than in female patients. One reason for these results was that the antipsychotic dose was substantially lower in female patients than in male patients (mean CP equivalent dose: 841 vs. 1066 mg/day; frequency of >1500 mg/day: 13.9% vs. 20.8%). In addition, because some previous studies in psychotic patients did not detect the gender difference (Chong et al., 2003; Hatta et al., 2000), such populations may have other factors that attenuate the gender difference.

There are several limitations to the study. First, we did not include medications other than psychotropic drugs in the analysis; however, the subjects included in the analysis were not coadministered other medical drugs that increased the risk for torsade de pointes (Chan et al., 2007). We also excluded patients suffering from cardiac diseases. Furthermore, psychotropic drugs that were administrated to 3% or fewer of the patients in the sample were not included in the analysis. The fact that nearly all patients received multiple drugs and a substantial proportion of participants (69%) were treated with antipsychotic polypharmacy may have made it difficult to obtain a clear result for each drug. However, there is great value in assessing the increased risk of QTc prolongation in such a practical setting. Our participants were all inpatients, and therefore individuals with severe symptomatology and those patients on high doses of antipsychotics were likely to be overrepresented. A recent study reported the possibility that an acute psychotic state itself may be a risk factor for QTc prolongation (Bar et al., 2007). Severe symptomatology might have biased the results toward an increased prevalence of the QTc interval in our subjects.

To screen QTc interval, we used an automated program, which may be fraught with errors. However, Charbit et al. (2006), for example, reported that patients with automatic QTc of <430 ms were at very low risk of having a prolonged QT interval where their definition of prolonged QTc interval was >450 ms in women and >440 ms in men. We measured QTc interval manually for patients with an automated QTc of >430 ms, although our definition of QTc prolongation was >480 ms in women and >470 ms in men. Thus, it was unlikely that we missed patients with QTc prolongation in our study. Furthermore, the reliability of the measurement algorithm of the ECG equipment (MAC 5500 with 12SL algorithm by GE health care [Amersham Place, Little Chalfont, Buckinghamshire, UK]) that we used was reported to be high. The data obtained by this algorithm was within 10 ms of the manual measurement in 95.9% of ECGs and within 15 ms in 99.3% of ECGs (Hnatkova et al., 2006). Thus, the possible effect of the use of the automated program is likely minimal. Another limitation might be that we used the chest lead with the maximal T-wave amplitude because clear T-wave leads are needed for precise

manual measurement. However, Bazett generally used limb lead II to determine his formula.

Despite these limitations, we obtained robust evidence among a large clinical sample in a real-world setting that suggested that a daily dose of antipsychotics is associated with a dose-dependent increased risk of QTc prolongation, whereas that of antiparkinsonian drugs, benzodiazepines, and mood stabilizers is not. With regard to individual antipsychotics, our results suggest that FGAs, such as HPDiv, CP, LP, and sultopride, have a risk-increasing effect on QTc prolongation and that SGAs, such as olanzapine, quetiapine, risperidone, and zotepine, are less likely to produce QTc prolongation than the FGAs. Such information may aid in clinical decision making concerning the choice of antipsychotic medication, particularly in patients who have an increased risk for arrhythmias.

5. Conclusions

We confirmed the statistical effect of chlorpromazine, levomepromazine, and HPDiv on QTc prolongation in a sample of 1017 patients with schizophrenia. Furthermore, statistical evidence for sultopride was obtained for the first time. Furthermore, in the range of the antipsychotic drugs that we examined, the data suggest that SGAs are less likely to produce QTc prolongation than FGAs, which may be useful in guiding the choice of antipsychotic drugs.

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Involvement of *SMARCA2*/BRM in the SWI/SNF chromatin-remodeling complex in schizophrenia

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Chromatin remodeling may play a role in the neurobiology of schizophrenia and the process, therefore, may be considered as a therapeutic target. The *SMARCA2* gene encodes BRM in the SWI/SNF chromatin-remodeling complex, and associations of single nucleotide polymorphisms (SNPs) to schizophrenia were found in two linkage disequilibrium blocks in the *SMARCA2* gene after screening of 11 883 SNPs (rs2296212; overall allelic $P = 5.8 \times 10^{-5}$) and subsequent screening of 22 genes involved in chromatin remodeling (rs3793490; overall allelic $P = 2.0 \times 10^{-6}$) in a Japanese population. A risk allele of a missense polymorphism (rs2296212) induced a lower nuclear localization efficiency of BRM, and risk alleles of intronic polymorphisms (rs3763627 and rs3793490) were associated with low *SMARCA2* expression levels in the postmortem prefrontal cortex. A significant correlation in the fold changes of gene expression from schizophrenic prefrontal cortex (from the Stanley Medical Research Institute online genomics database) was seen with suppression of *SMARCA2* in transfected human cells by specific siRNA, and of orthologous genes in the prefrontal cortex of *Smarca2* knockout mice. *Smarca2* knockout mice showed impaired social interaction and prepulse inhibition. Psychotogenic drugs lowered *Smarca2* expression while antipsychotic drugs increased it in the mouse brain. These findings support the existence of a role for BRM in the pathophysiology of schizophrenia.

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INTRODUCTION

Schizophrenia is a chronic, severe and disabling brain disorder that affects approximately 1% of the world's population. A large body of data consistently supports the involvement of complex genetic components causally linked to schizophrenia. Association studies including genome-wide scans have identified many risk alleles that have small effects (1–5). In addition to genetic studies, altered expression of many genes and proteins in schizophrenic brains has been documented (6) and epigenetic regulation in schizophrenia has also been studied (7). Among epigenetic mechanisms, the role of chromatin modification in psychiatric disorders and related fields has been reported for epilepsy (8), drug addiction (9,10), depression (11), autism (12), fear (13), learning and memory (13,14), social cognition (15) and stress-related behaviors (16,17). Histone modifications may contribute to the pathogenesis of prefrontal dysfunction in schizophrenia (18).

The *SMARCA2* gene encodes BRM, one of the earliest described chromatin remodeling multiprotein complexes in the yeast SWI/SNF complex (19–23), and highly conserved among eukaryotes (24). The SWI/SNF molecules are mutually exclusive within the complexes and harbor ATPase activity (25–27). This complex functions by destabilizing the interactions between DNA and histones in the nucleosome in an ATP-dependent reaction (28,29). Mammalian SWI/SNF complexes are present in biochemically diverse forms, indicating that they may have specialized nuclear functions (30). ATP-dependent remodeling complexes involved in chromatin opening or compaction are important in the regulation of transcriptional processes associated with development, cellular differentiation and oncogenesis (22,31–35).

BRM interacts with several transcription factors and other DNA-binding proteins and is involved in chromatin structural modification in the epigenetic regulation of gene expression (36). *SMARCA2* expression is induced to a high level during differentiation to neurons and astrocytes, suggesting an important role in neural cell differentiations (37). Because BRM potentially influences expression of many genes, it is hypothesized that functional changes to *SMARCA2*/BRM may contribute to gene expression changes reported to occur in schizophrenia.

RESULTS

SNPs in two linkage disequilibrium blocks within the SMARCA2 gene were associated with schizophrenia in the Japanese population

Initially, in 100 Japanese schizophrenic patients, 11 883 SNPs were screened for association with schizophrenia. These SNPs exist on the Illumina Human-1 BeadChip and are also deposited in a Japanese SNP (JSNP) database of 1480 Japanese control chromosomes (http://snp.ims.u-tokyo.ac.jp/index.html). The potential impact of population structure on this association study was evaluated by using the genome-wide χ^2 inflation factor, λ, as a genomic control (38,39). The estimated value of λ was 1.05, by which genome-wide association *P*-values were corrected. After correction for multiple testing of these 11 883 SNPs, no significant association was found between them and schizophrenia (Supplementary Material, Table S1).

In the replication 1 cohort, comprised of 576 Japanese schizophrenic patients and 576 Japanese control subjects, an attempt was made to replicate the association of the top 5 SNPs, as ranked by the association *P*-values, with schizophrenia. A potential association was found for only one, rs2296212 (one-sided allelic $P = 0.009$) (Supplementary Material, Table S1). Subsequent analysis in the replication 2 samples of 1344 Japanese schizophrenic patients and 1344 Japanese control subjects confirmed the association for this SNP (one-sided allelic $P = 0.04$). The *P*-value for the association was 5.8×10^{-5} when the initial genome-wide sample and the total replication 1 + 2 samples were combined (Table 1).

Because the *SMARCA2* gene encodes BRM in SWI/SNF chromatin remodeling, SNPs within genes that encode proteins potentially involved in chromatin remodeling were re-evaluated for association with schizophrenia. *CREBBP, DNMT1, DNMT2, DNMT3A, DNMT3B, HAT1, HDAC2, HDAC3, HDAC4, HDAC7, HDAC9, HNMT3A, MYST1, MYST2, MYST4, SIN3A, SIN3B, SMARCA3, SMARCA4, SMARCA5* and *SMARCC1* were selected in addition to the *SMARCA2* genes, with a less stringent criteria of association used (*P*-values of less than 0.05). For this screen, SNPs from the same 100 schizophrenia patients were screened with an Illumina HumanHap370 BeadChip and compared with those in 1868 chromosomes of Japanese volunteers listed in the JSNP database [deposited October 2007, (http://snp.ims.u-tokyo.ac.jp/index.html)]. Although potentially significant association was observed in the *SMARCC1* and *DNMT3B* genes, this did not exist after correction for multiple testing (Supplementary Material, Table S2). An attempt was made to replicate the associations of rs13063042 and rs17079785 in *SMARCC1* and rs2424932 in *DNMT3B* with schizophrenia in the replication 1 sample. However, no significant results were obtained (Supplementary Material, Table S2).

The *SMARCA2* gene is located at chromosome 9p24.3 and spans 178 282 bp comprising 34 exons. The SNP of rs2296212 was non-synonymous, D1546E, in exon 33. Subsequent genotyping of 34 tag SNPs in the *SMARCA2* gene in the replication 1 + 2 samples identified a significant association with schizophrenia for three SNPs [rs2066111 in intron 12 (allelic $P = 8.2 \times 10^{-5}$), rs3763627 in intron 12 (allelic $P = 1.2 \times 10^{-5}$) and rs3793490 in intron 19 (allelic $P = 3.0 \times 10^{-6}$), Fig. 1 and Table 1]. Resequencing of the coding region of *SMARCA2* in 24 Japanese patients with schizophrenia identified no non-synonymous mutations except for D1546E.

The distance between D1546E and rs3793490 was 95 351 bp and these two SNPs were not in linkage disequilibrium ($r^2 = 0$, $D' = 0$–0.16). The three intronic SNPs were in modest linkage disequilibrium with each other; the *r* squares were 0.59 between rs2066111 and rs3763627, 0.52 between rs206111 and rs3793490 and 0.85 between rs3763627 and rs3793490. Therefore, SNPs in two linkage disequilibrium blocks in the *SMARCA2* gene, one in the middle and one in the 3′ region of the gene, were found to be associated with schizophrenia in our Japanese population.

Table 1. Associations between SNPs in the SMARCA2 gene and schizophrenia in the Japanese populations

SNP ID	Population		Genotype count (frequency)			P	Allele count (frequency)		P	Odds ratio (95% CI)
rs2296212			CC	CG	GG		C	G		
	Affected (screening)	$n = 100$	59 (0.59)	32 (0.32)	9 (0.09)		150 (0.75)	50 (0.25)		1.90 (1.34–2.69)
	Controls (JSNP)[a]	$n = 750$					1276 (0.85)	224 (0.15)	0.0003	
	Affected (replication 1)	$n = 572$	370 (0.65)	168 (0.29)	34 (0.06)		908 (0.79)	236 (0.21)		1.29 (1.05–1.60)
	Controls (replication 1)	$n = 574$	398 (0.69)	160 (0.28)	16 (0.03)	0.02	956 (0.83)	192 (0.17)	0.009[b]	
	Affected (replication 2)	$n = 1343$	907 (0.68)	384 (0.29)	52 (0.04)		2198 (0.82)	488 (0.18)		1.14 (0.99–1.31)
	Controls (replication 2)	$n = 1338$	933 (0.70)	373 (0.28)	32 (0.02)	0.07	2239 (0.84)	437 (0.16)	0.04[b]	
	Affected (replication 1 + 2)	$n = 1915$	1277 (0.67)	552 (0.29)	86 (0.04)		3106 (0.81)	724 (0.19)		1.18 (1.05–1.33)
	Controls (replication 1 + 2)	$n = 1912$	1331 (0.70)	533 (0.28)	48 (0.03)	0.005	3195 (0.84)	629 (0.16)	0.0027[b]	
	Overall (screening+replicatinon)	$n = 2015$	1336 (0.66)	584 (0.29)	95 (0.05)		3256 (0.81)	74 (0.19)		1.25 (1.12–1.39)
	Overall (JSNP+replication)						4471 (0.84)	853 (0.16)	5.8×10^{-5}	
rs2066111			GG	AG	AA		G	A		
	Affected (replication 1 + 2)	$n = 1912$	974 (0.51)	776 (0.41)	162 (0.08)		2724 (0.71)	1100 (0.29)		1.22 (1.10–1.34)
	Controls (replication 1 + 2)	$n = 1907$	846 (0.44)	866 (0.45)	195 (0.10)	8.0×10^{-5}	2558 (0.67)	1256 (0.33)	8.2×10^{-5}	
rs3763627			AA	AT	TT		A	T		
	Affected (replication 1 + 2)	$n = 1905$	1116 (0.59)	690 (0.36)	99 (0.05)		2922 (0.77)	888 (0.23)		1.26 (1.14–1.40)
	Controls (replication 1 + 2)	$n = 1900$	1002 (0.53)	744 (0.39)	154 (0.08)	1.0×10^{-5}	2748 (0.72)	1052 (0.28)	1.2×10^{-5}	
rs3793490			GG	GT	TT		G	T		
	Affected (screening)	$n = 122$	9 (0.07)	37 (0.30)	76 (0.62)		55 (0.23)	189 (0.77)		1.29 (0.94–1.77)
	Controls (JSNP)	$n = 934$	83 (0.09)	344 (0.37)	507 (0.54)	0.13	510 (0.27)	1358 (0.73)	0.11	
	Affected (replication 1 + 2)	$n = 1895$	109 (0.06)	688 (0.36)	1098 (0.58)		906 (0.24)	2884 (0.76)		1.27 (1.15–1.41)
	Controls (replication 1 + 2)	$n = 1908$	162 (0.08)	767 (0.40)	979 (0.51)	4.0×10^{-6}	1091 (0.29)	2725 (0.71)	3.0×10^{-6}	
	Overall (screening+replicatinon)	$n = 2017$	118 (0.06)	725 (0.36)	1174 (0.58)		961 (0.24)	3073 (0.76)		1.25 (1.14–1.38)
	Overall (JSNP+replication)	$n = 2842$	245 (0.09)	1111 (0.39)	1486 (0.52)	2.0×10^{-6}	1601 (0.28)	4083 (0.72)	2.0×10^{-6}	

Allelic P-values were calculated by chi-square test (two-sided) and genotypic P-values were Cochran-Armitage test.
[a]http://snp.ims.u-tokyo.ac.jp/cgi-bin/SnpInfo.cgi?SNP_ID=IMS-JST050328.
[b]One-sided.

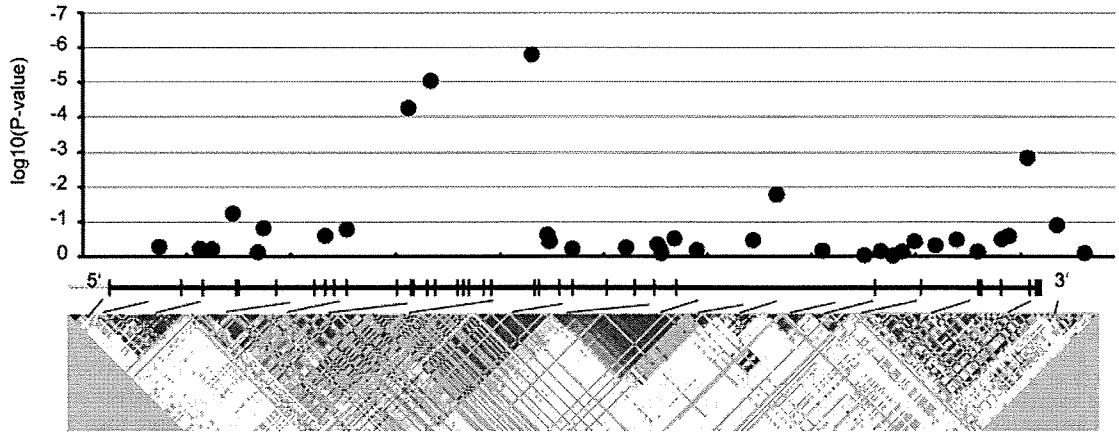

Figure 1. Association of tag SNPs in *SMARCA2* with schizophrenia in the replication samples. Linkage disequilibrium in the HapMap data is also shown with red indicating high linkage disequilibrium and white denoting low linkage disequilibrium. Exons are shown in the middle.

Risk alleles of intronic SNPs were associated with lower *SMARCA2* gene expression level in the postmortem prefrontal cortex

Transcription level in the postmortem prefrontal cortex, as measured by TaqMan real-time PCR, was not significantly different by diagnosis, ethnicity, age, sex, postmortem intervals (PMI) or pH of brain samples. Linear multiple regression analysis including sex, age, PMI, pH, ethnicity, diagnosis and genotypes of the SNPs present resulted in a significant associations of rs3763627 ($P = 0.005$) and rs3793490 ($P = 0.01$) with SMARCA2 expression level. The difference in *SMARCA2* expression level between the genotypes is shown in Figure 2. Alleles observed more frequently in the schizophrenia group were associated with a low expression level of *SMARCA2* in the prefrontal cortex. When Australian and Japanese schizophrenic subjects, or schizophrenia patients and controls were separately analyzed, there were no significant differences seen in *SMARCA2* expression level. *SMARCA2* expression level was not significantly different between the genotypes of rs2066111 and rs2296212.

The risk allele E1546 causes lower nuclear localization efficiency of BRM

BRM has several domains highly conserved among species, such as yeast (23), Drosophila (40) and mammals (27). Although D1546E is located downstream to the bromodomain, the polymorphism is in the highly conserved region among mammalian species, and glutamic acid (E) at the 1546 bp site in human is commonly found among mammalian species (Fig. 3A).

BRM is localized in the nucleus (19). In order to analyze any functional difference of BRM between the two allele types, localization of BMR was investigated by EGFP fusion protein (Fig. 3B) transfected into the human glioblastoma cell line T98G. The D1546 type of EGFP fused BRM (EGFP/BRM*D) localized to the nucleus; however, the E1546 type of EGFP fused BRM (EGFP/BRM*E) existed both in the cytoplasm and nucleus (Fig. 3C and D). Cells transfected with the two different alleles had a different morphology (Fig. 3C). This finding indicates lower nuclear localization efficiency of the E1546 isoform (BRM*E) in transfected cells, and it is hypothesized that the E1546 isoform has less functionality than the D1546 type (BRM*D).

Lower function of the E1546 form of BRM was supported by transcriptional changes seen in transfected cells

The functional capability of BRM*E was evaluated by comparisons of gene expression changes that were introduced by *SMARCA2**E, *SMARCA2**D and siRNA targeted towards SMARCA2. pDEST26 expression vectors were constructed with *SMARCA2**E or *SMARCA2**D and introduced into T98G cells. Transcription of *SMARCA2**E and *SMARCA2**D was 50-folds higher than that of control cells (Fig. 4A). The siRNA targeted against the *SMRACA2* gene was introduced into the same cell lines and translation level decreased to approximately 1/10 of that from control cells (Fig. 4A). Transcription levels of *SMARCA2* were comparable to the level of translation seen after the immunoblot analysis (Fig. 4A). After transfection of the pDEST26 with *SMARCA2*E*, the pDEST26 with *SMARCA2*D*, and siRNA in T98G cells, gene expression level was measured using Sentrix Human WG-6 BeadChips (Illumina, CA, USA). Expression changes introduced by *SMARCA2*E*, compared with that of *SMARCA2*D*, were significantly correlated with those of the siRNA treatment, compared with that of mock-treated cells (Fig. 4B, $P < 0.0001$), again supporting a lower functionality of BRM*E compared with BRM*D. When outliers defined as values exceeding two standard deviations from the mean in *SMARCA2**E against *SMARCA2**D, and from that in siRNA were excluded, the correlation was more significant ($P < 0.00001$).

Gene expression changes seen after suppression of SMARCA2 in transfected human cells were correlated with those found in the postmortem prefrontal schizophrenic brains

To evaluate the relationship between lower functioning of SMARCA2 and the gene expression profile seen in schizophrenia, gene expression changes after introduction of

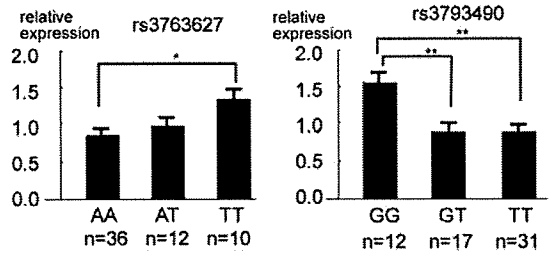

Figure 2. *SMARCA2* expression levels in the postmortem prefrontal region by genotype [rs3763627 (intron 12) and rs3793490 (intron 19)]. The symbols * and ** indicate $P < 0.05$ and $P < 0.01$, respectively, by Student's *t*-test. The A allele of rs3763627 ($P = 0.005$) and the T allele of rs3793490 ($P = 0.01$) were associated with lower expression level, when genotype was coded as 0, 1 or 2 depending on the number of copies of the risk allele present, and multiple a simple regression model was fitted. The vertical scores show average (SEM) of relative expression in each of the three genotype groups, compared with mean gene expression in the total samples.

siRNA to T98G cells were compared with those from the postmortem prefrontal cortex of schizophrenia patients. Data from all frontal cortex studies were utilized to determine the mean expression of each gene. Among 1051 genes with significant expression changes seen in schizophrenic patients from the SMRI database ($P < 0.05$, SMRIDB, https://www.stanleygenomics.org/), 445 genes could not be evaluated due to their low expression level in T98G cells. The fold change of expression of the remaining 606 genes from the SMRIDB was significantly correlated with expressional changes seen after siRNA treatment in T98G cells ($P < 0.0009$) (Fig. 5A). When outliers defined as mean values exceeding 10-folds in siRNA against mock were excluded, the correlation was still significant ($P < 0.003$).

Gene expression profiles from *Smarca2* knockout mice prefrontal cortex were correlated with those seen in the postmortem prefrontal schizophrenic brains

To evaluate the relationship between lower functionality of BRM and the gene expression profile of schizophrenia, gene expression profiles from the prefrontal cortex of *Smarca2* knockout mice (41) were compared with those from the postmortem prefrontal cortex of schizophrenic patients in the SMRIDB. Gene expression in the prefrontal cortex of three pairs of *Smarca2* −/− mice and littermate wild-type mice (*Smarca2* +/+) at 8 weeks of age were measured with MouseWG-6 BeadChips (Illumina, CA, USA). Expression level of 586 genes was significantly different between *Smarca2* −/− mice and *Smarca2* +/+ mice (*t*-test, $P < 0.05$). Fifty-two orthologous genes were found between these 586 genes and the 1051 genes with significant expression changes seen from schizophrenic patients compared with controls in the SMRIDB (Table 2). The fold change in expression of these 52 genes in *Smarca2* −/− mice compared with *Smarca2* +/+ mice was significantly correlated with the fold changes of orthologous genes in schizophrenia compared with controls in the SMRIDB ($P < 0.002$) (Fig. 5B). When outliers defined as mean values in *Smarca2* −/− mice exceeding 2-folds from the mean in *Smarca2* +/+ mice were excluded, the correlation was more significant ($P < 0.001$).

Confirmation of genes interacting with BRM by ChIP assay

Although transcriptional changes were observed in many genes by down- or up-regulation of the *SMACA2* gene in cultured T98G cells or in the *Smarca2* knockout prefrontal brain, they were direct or indirect consequences of interaction with the BRM-containing SWI/SNF chromatin remodeling factors. To confirm the association of endogenous BRM with the promoters of these transcriptionally influenced genes, ChIP assay was carried out using an antibody against BRM. DNA regions that interacted with BRM were collected and the sequence for −1163 to −1026 bp up-stream of the *HOMER1* gene confirmed by PCR. The *HOMER1* gene was selected because, among the genes in Table 2, it exhibited a more than 10-fold reduction in the expression in T98G cells after transfection by the siRNA targeted towards *SMARCA2*, and a more than 10-fold increase after transfection of the pDEST26 with *SMARCA2*E* or the pDEST26 with *SMARCA2*D*. These regions were also detected after ChIP assay using an antibody against MeCP2, which interacted with BRM (Fig. 6).

Impaired social interaction and prepulse inhibition in *Smarca2* knockout mice

To evaluate schizophrenia-related behaviors in *Smarca2* knockout mice, social interaction and prepulse inhibition (PPI) of the acoustic startle reflex was measured. Male and female data were combined for the analysis because no sex differences were observed. *Smarca2* −/− mice spent a significantly shorter time when making contact with an unfamiliar intruder mouse compared with mice of the other genotypes (Fig. 7A, $P = 0.03$). There was no significant difference in novelty seeking behavior between genotypes (data not shown). *Smarca2* −/− mice showed significant disturbance of PPI at 78 dB ($P = 0.02$) and a trend toward disturbance of PPI at 82 dB and 86 dB ($P = 0.07$) compared with the other genotypes (Fig. 7B).

Smarca2 gene expression in the mouse brains was decreased by psychotogenic drugs treatments and increased by antipsychotic drug treatments

The involvement of aberrant NMDA receptor signaling and a hyperdopaminergic state has been assumed in the pathophysiology of schizophrenia. *Smarca2* expression was evaluated in the mouse brain using the MK-801 non-competitive antagonist of NMDA receptors, the indirect dopamine receptor agonist methamphetamine and the antipsychotic drugs haloperidol and olanzapine. The expression of *Smarca2* was significantly decreased after the administration of MK-801 or methamphetamine and increased by administration of haloperidol and olanzapine (Fig. 7C).

DISCUSSION

SNPs in two linkage disequilibrium blocks in the *SMARCA2* gene were associated with schizophrenia in Japanese populations and the risk alleles are likely to confer a lower functioning of *SMARCA2*/BRM through altered gene

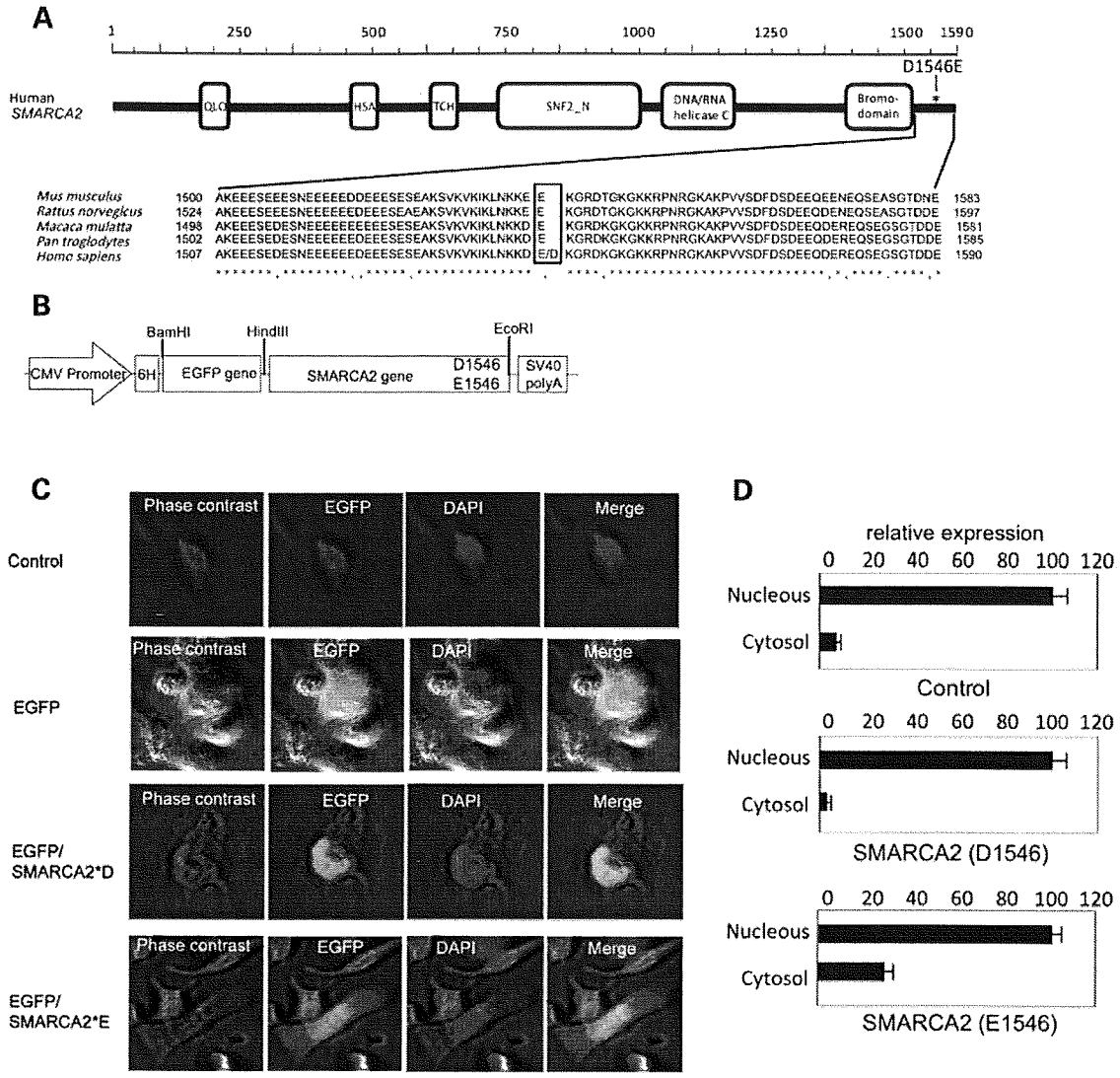

Figure 3. Nuclear localization efficiency of BRM between alleles of rs2296212 (D1546E). (**A**) Domains in BRM and evolutional comparisons of sequences around human D1546E. QLQ, Gln-Leu-Gln motif; HAS, Helicase/SANT-associated, DNA binding; TCH, associated with TFs and helicases; DNA/RNA helicase C; Bromodomain, an acetyl-lysine binding domain. Arrow indicates the position of the D1546E polymorphism. (**B**) Plasmid construction for the fusion proteins. CMV pro., cytomegalovirus promoter; 6H, 6 histidine; EGFP, enhanced green fluorescence protein; SMARCA2; SV40 poly A, SV40 polyadenylation signal. (**C**) Different cell morphology and intracellular localization of EGFP-SMARCA2 fusion protein between D1546 and E1546 forms in transfected T98G human glioblastoma cells. Control: a cell transfected with pDEST26 vector; EGFP: cells transfected with pDEST26 with EGFP; EGFP/SMARCA2*D: a cell transfected with pDEST26 EGFP-SMARCA2 (D1546); EGFP/SMARCA2*E: cells transfected with pDEST26 EGFP-SMARCA2 (E1546). One hundred cells were visualized for each sample. (**D**) Quantification of BRM expression in nucleus and cytosol of cells by western blot analysis. Protein expression level is shown as a mean (SEM) ratio of the protein expression level in the nucleus. Data from triplicate experiments were normalized to the expression of β-actin.

expression or intracellular localization. Although the study did not find significant differences in *SMARCA2* transcription levels in the postmortem prefrontal cortex between schizophrenic patients and controls (data not shown), the SMRI database showed a non-significant trend toward decreased *SMARCA2* transcription levels in schizophrenics compared with controls ($P = 0.07$). Additionally, a disruption of the *SMARCA2* gene in a patient with schizophrenia has been reported (42). BRM is involved in the modification of chromatin structures in epigenetic regulation of gene expression (36). Therefore, it was hypothesized that low functionality of *SMARCA2*/BRM is associated with schizophrenia through its pleiotropic effects on transcriptional regulation of many genes. This hypothesis is supported by gene expression profiles from the prefrontal cortex and behavioral observations in *Smarca2* knockout mice.

From an evolutionary perspective, the amino acid residue corresponding to human D1546E in *SMARCA2* (*Smarca2*) of dog, mouse, rat, horse, cow and chimpanzee is E. Therefore, E1546 is probably the ancestral type in humans (Fig. 3A).

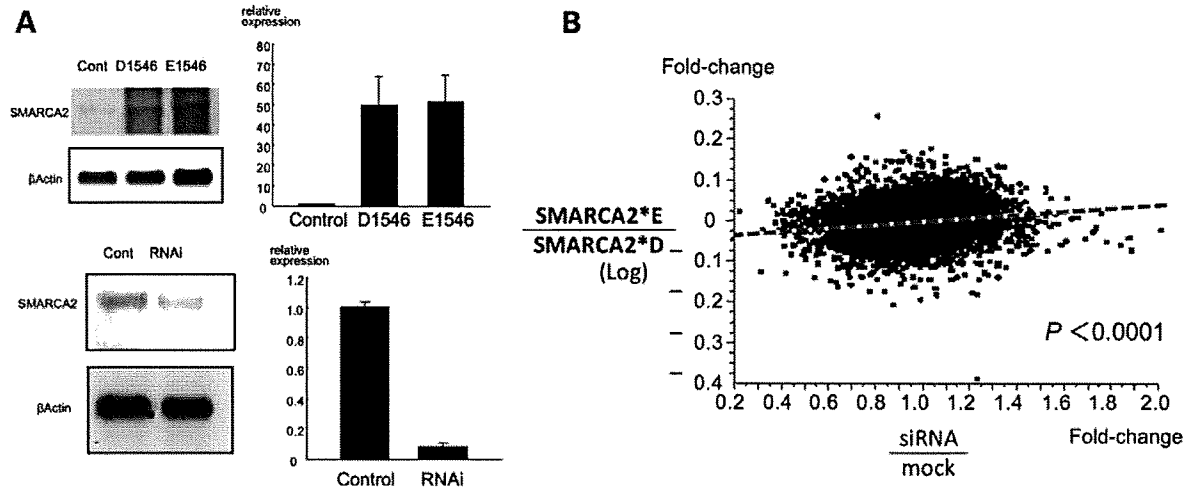

Figure 4. Evaluation of the E1546 and D1546 forms of BRM by gene expression profiles. (**A**) The expression levels of *SMARCA2* in T98G cells transfected by the pSMARCA2*E and *D constructs detected by northern blotting (upper-left) and real-time PCR (upper-right). For real-time PCR, the expression ratio is shown using the transcription level of a control as 1. The expression levels of *SMARCA2* gene knocked down with siRNAs by northern blotting (lower-left) and real-time PCR (lower-right). The data from triplicate experiments were normalized to the expression of *β-actin* or *GAPDH* gene. (**B**) Regression analysis between fold difference in expression in T98G cells transfected with pDEST26 with *SMARCA2*E*, cells transfected with pDEST26 with *SMARCA2*D* and cells transfected with siRNA compared with mock transfected cells. Gene expression after transfection was measured with Sentrix Human WG-6 BeadChips (Illumina).

Because the allele frequencies of D1546 were 0.85 in Japanese, 0.75 in Yoruba and 0.9 in CEPH families (according to the HapMap data), it might be assumed that D1546, the protective allele for schizophrenia, arose during the human evolutionary process and spread due to its positive selection pressure against schizophrenia.

The second finding of the present study is that BRM is a potential key protein in schizophrenia. Functional differences between alleles in humans are likely to be small, as indicated by the relatively small odds ratios of the risk alleles observed in Japanese populations (1.18 to 1.27). Therefore, the contribution of genetic variations in the *SMARCA2* gene region to the development of schizophrenia may be small. However, the present study suggests a greater role of the *SMARCA2* gene than the genetically determined role in the pathophysiology and amelioration of schizophrenia because psychotogenic drugs (well-established pharmacological models of schizophrenia) decreased *Smarca2* expression and an antipsychotic drug increased expression in the mouse brain. These findings support the hypothesis of BRM as being a potential key molecule involved in schizophrenia. The hypothesis is that various psychotogenic factors including genetic ones decrease BRM, which further affects expression of various other genes that then contribute to the development of schizophrenia.

BRM is involved in the epigenetic mechanisms of psychotogenic and antipsychotic drugs. As for the relationship of these drugs with epigenetic mechanisms, the influences of methamphetamine on DNA methyltransferase mRNA levels (43,44) and that of D2-like antagonists and MK-801 on the phosphorylation of histone H3 at serine 10 and the acetylation of H3-lysine 14 (45) have been reported. The present study also indicates the involvement of the SNF/SWI family protein in the epigenetic mechanisms through which psychotogenic and antipsychotic drugs act.

The statistical evidence in the present study should be considered cautiously given that genotyping was based upon different platforms performed in different laboratories for screening with the Illumina BeadChips. This could likely result in false positives. Therefore, importance was placed on real statistical support from the replication performed. However, the initial screening using 11 883 SNPs and 100 schizophrenia patients is far from a complete genome coverage and has an extremely low power to detect a true association. This may affect the credibility of the results. Confirmation of associations in populations other than Japanese is necessary.

There are many questions yet to be answered. The mechanisms of influences of psychotogenic and antipsychotic drugs on *SMARCA2* expression and the mechanisms of regulation of each gene listed in Table 2 involving BRM are unknown. An interaction between the promoter region of the *HOMER1* gene and BRM was confirmed using the ChIP assay. HOMER proteins provide constitutive forms of Homer (also known as CC-Homers) and immediate early gene products. Homer proteins interact with both group 1 metabotropic glutamate receptors (mGluRs) of mGluR1 and mGluR5 (46) and Shank-GKAP-PSD95-NMDA receptor complexes, as well as with proteins that regulate intracellular calcium signaling. *Homer1* knockout mice also show behavioral and neurochemical phenotypes relevant to schizophrenia (47,48).

The finding that psychotogenic drugs decreased Smarca2 expression can be interpreted as suggesting that changes in *SMARCA2* are consequences of schizophrenia rather than causes. Although genetic association in humans and altered behavior in Smarca2 knockout mice may support causation at least in part, the experiments from this study indicate the existence of factors that influence SMARCA2 expression

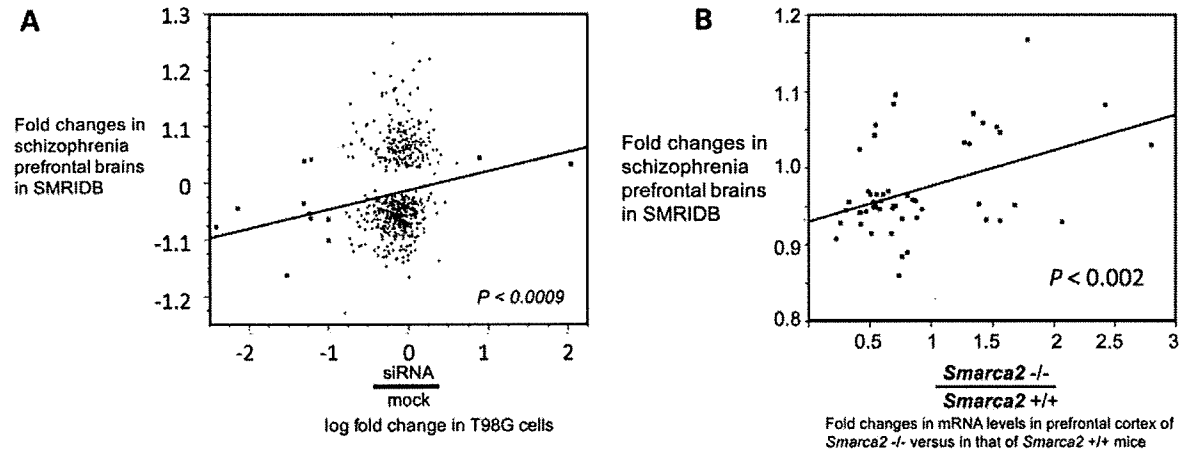

Figure 5. Correlation of transcriptional fold changes in the postmortem prefrontal cortex in SMRIDB with those in T98G cells after siRNA treatments (**A**) and with *Smarca2* knockout prefrontal mouse brains (**B**). Transcriptional fold changes by siRNA against mock in T98G cells were log-transformed (A). A simple regression analysis was carried out.

and may be related to schizophrenia. Further studies exploring such factors are warranted.

Frequent loss of BRM expression has been reported in lung cancers (49) and gastric cancer (50) and a lower average level of BRM expression in prostate cancers (51). Controversy concerning the incidence of cancer in schizophrenia exists; however, lower respiratory (52) and prostate cancers (53) in schizophrenia patients have been reported. A recent meta-analysis indicated a slightly increased incidence of lung cancer in schizophrenic patients, but after the data were adjusted for smoking prevalence, this was not seen (54).

In conclusion, the present study identifies BRM as potentially a key molecule in a wide range of pathophysiology associated with schizophrenia.

MATERIALS AND METHODS

Human subjects

Subjects of schizophrenia for screening, replication 1 and replication 2 were 100 (mean age ± SD: 57.5 ± 14.9 years, 58 males and 42 females), 576 (mean age ± SD: 51.6 ± 14.8 years, 322 males and 254 females) and 1344 (mean age ± SD: 46.7 ± 14.4 years, 733 males and 611 females) and control subjects in replications 1 and 2 were 576 (mean age ± SD: 46.8 ± 12.5 years, 268 males and 322 females) and 1344 (mean age ± SD: 47.8 ± 13.8 years, 783 male and 561 female). The replication samples were independent from the sample set used for screening. For every possible paring of individuals, the mean and variance in number of alleles shared identity-by state (IBS) across markers was estimated using the GRR tool (55). All subjects were of Japanese descent and were recruited from the main island of Japan. All schizophrenic subjects were given a best-estimate lifetime diagnosis according to DSM-IV criteria with obtained consensus from at least two experienced psychiatrists on the basis of all available sources of information, including unstructured interviews, clinical observations and medical records. Control subjects were mentally healthy and had no family history of mental illness within second-degree relatives as self-reported. The study was approved by the Ethics Committees of the University of Tsukuba, Niigata University, Fujita Health University, Nagoya University, Okayama University and Seiwa Hospital, and all participants provided written informed consent.

Postmortem brains

Brain specimens were from European-descent Australian individuals and Japanese individuals. The Australian sample comprised 10 schizophrenic patients and 10 age- and gender-matched controls (56). The diagnosis of schizophrenia had been made according to the Diagnostic and Statistical Manual of Mental Disorders (DSM-IV) criteria (American Psychiatric Association 1994) by a psychiatrist and a senior psychologist. Control subjects had no known history of psychiatric illness. Tissue blocks were cut from gray matter in an area of the prefrontal cortex referred to as Brodmann's area 9 (BA9). Japanese samples of BA9 gray matter were from 6 schizophrenic patients and 11 age- and gender-matched controls (56). In addition, postmortem brains of 37 deceased Japanese patients with schizophrenia were also analyzed (56). The Japanese subjects met the DSM-III-R criteria for schizophrenia. The study was approved by the Ethics Committees of the Central Sydney Area Health Service, University of Sydney, Niigata University, University of Tsukuba, Tokyo Metropolitan Matsuzawa Hospital and the Tokyo Institute of Psychiatry.

Genotyping

Association screening was performed using the Illumina Sentrix Human-1 Genotyping 109 k BeadChip and HumanHap370 BeadChip according to the manufacturer's instructions (Illumina, San Diego CA, USA). All DNA samples were subjected to rigorous quality control to check for fragmentation and amplification. Approximately 750 ng of

Table 2. Significantly differently expressed genes in the prefrontal cortex of *Smarca2* knockout mice and in the postmortem prefrontal cortex of schizophrenia in Stanley database

Genes (human)	Stanley SZ against control Gene expression fold change	StanleyP	Genes (mouse)	accession ID	*Smarca2* −/− against +/+ Gene expression fold change
APPBP2	−1.0335	0.042	*Appbp2*	*NM_025825.2*	−1.54
ARHGEF9	−1.0579	0.008	*Arhgef9*	*NM_025657.2*	−1.46
ARPP-19	−1.0632	0.045	*Arpp-19*	*NM_030562.1*	−2.32
ASPH	1.0313	0.012	*Asph*	*NM_173382*	1.27
ASPSCR1	−1.0471	0.045	*Aspscr1*	*NM_144960.1*	−1.69
BDNF	−1.0523	0.031	*Bdnf*	*NM_007540.3*	−1.82
CDK5R2	−1.0617	0.021	*Cdk5r2*	*NM_177775.2*	−2.11
CLCN3	−1.0562	0.020	*Clcn3*	*NM_183108.1*	−1.89
CPSF6	−1.0377	0.047	*Cpsf6*	*NM_017372.2*	−1.79
CSDA	1.0577	0.030	*Csda*	*NM_028878.1*	1.43
CXCR4	−1.0757	0.002	*Cxcr4*	*XM_130951.1*	1.57
DOCK11	−1.0530	0.003	*Dock11*	*NM_001033349.1*	1.69
DOK5	−1.0952	0.007	*Dok5*	*NM_007386.1*	−1.93
DUSP3	−1.0795	0.001	*Dusp3*	*NM_025657.2*	−3.72
ERCC1	−1.0549	0.045	*Ercc1*	*NM_008701.1*	−1.43
FKBP5	1.0521	0.046	*Fkbp5*	*NM_007386.1*	1.54
GPAA1	−1.0745	0.005	*Gpaa1*	*NM_138648.1*	1.45
GSTT2	−1.0770	0.015	*Gstt2*	*NM_011986.2*	2.07
HAGHL	−1.1255	0.005	*Haghl*	*NM_139064.1*	−1.24
HDLBP	−1.0482	0.007	*Hdlbp*	*NM_013881.3*	−3.00
HGF	1.0233	0.005	*Hgf*	*NM_028094.1*	−2.39
HOMER1	−1.1043	0.017	*Homer1*	*NM_147176.1*	−4.39
HRK	−1.0488	0.040	*Hrk*	*NM_028094.1*	−1.88
IFITM2	1.1655	0.001	*Ifitm2*	*NM_028878.1*	1.79
IGFBP7	1.0935	0.000	*Igfbp7*	*XM_203293.2*	−1.41
KCNK1	−1.1331	0.001	*Kcnk1*	*NM_013881.3*	−1.30
LGI4	1.0299	0.039	*Lgi4*	*NM_011986.2*	1.32
MAPK8	−1.0373	0.033	*Mapk8*	*NM_028094.1*	−1.65
MAPK9	−1.0640	0.038	*Mapk9*	*NM_011719.2*	−2.38
NAP1L1	−1.0396	0.009	*Nap1l1*	*NM_026000.1*	−1.24
OSBP	−1.0512	0.017	*Osbp*	*NM_130859.2*	1.39
PER1	1.0806	0.006	*Per1*	*XM_129848.4*	2.42
PINK1	−1.0958	0.012	*Pink1*	*NM_011960.1*	−1.47
POU2F1	1.0695	0.044	*Pou2f1*	*NM_007386.1*	1.35
PTBP1	1.0286	0.048	*Ptbp1*	*NM_027563.1*	2.81
PTPRB	−1.0340	0.026	*Ptprb*	*NM_001033349.1*	−2.03
RAB1A	−1.0540	0.029	*Rab1a*	*NM_009695.2*	−1.40
RNF14	−1.0818	0.002	*Rnf14*	*NM_153415.1*	−2.32
RTN4	−1.0584	0.050	*Rtn4*	*NM_138648.1*	−1.72
RUFY2	1.0410	0.020	*Rufy2*	*NM_153415.1*	−1.85
SGC2	−1.1655	0.002	*Sgc2*	*NM_009129.1*	−1.36
SDC2	1.0811	0.001	*Sdc2*	*NM_008701.1*	−1.44
SDHC	−1.0471	0.045	*Sdhc*	*NM_011762.2*	−1.14
SFRS11	1.0537	0.011	*Sfrs11*	*XM_135197.4*	−1.82
SNX4	−1.0385	0.034	*Snx4*	*NM_177775.2*	−1.96
SRR	−1.0459	0.028	*Srr*	*NM_030562.1*	−1.17
SYT11	−1.0715	0.024	*Syt11*	*NM_009759.2*	−1.13
TNFRSF25	1.0440	0.014	*Tnfrsf25*	*NM_028878.1*	1.56
TPI1	−1.0590	0.018	*Tpi1*	*NM_001013823.1*	−1.08
UBXD1	−1.0728	0.040	*Ubxd1*	*NM_153415.1*	−1.31
VAMP1	−1.0563	0.034	*Vamp1*	*NM_177775.2*	−1.81
WARS	−1.0604	0.050	*Wars*	*NM_001033349.1*	−3.25

Stanley-*P*-values are by the SMRI database.

genomic DNA was used in each sample. Normalized bead intensity data obtained for each sample were entered into the Illumina BeadStudio 3.0 software, which converted fluorescence intensities into SNP genotypes. A GenCall Score of 0.85 was used as a minimum threshold for per-sample genotyping completeness. The mean call rate across all samples was 97.0% for Human-1 and 99.8% for HumanHap370; the call rate was at least 99% for 47021 SNPs for Human-1 and 235868 SNPs for HumanHap370 and at least 95% for 60 568 SNPs for Human-1 and 244 337 for HumanHap370. Concordance rate between Human-1 and HumanHap370 platforms was evaluated by comparisons of genotypes in the 100 screening samples and this gave concordance of over 98.0% for each sample. One thousand one hundred and fifty-two subjects were genotyped twice for each SNP using TaqMan genotyping (Applied Biosystems, Foster City, CA, USA),

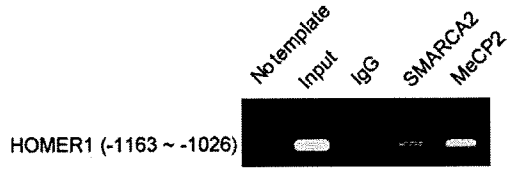

Figure 6. Chromatin immunoprecipitation (ChIP) assays on DNA harvested from T98G cells. DNA that interacts with SMARCA2 and MeCP2 was evaluated using antibodies against to them. Normal rabbit immunoglobulin G (IgG) was used as negative controls. DNA was detected by PCR using primers for the region −1163∼−1036 from the *HOMER1* exon 1. 1st lane: no template; 2nd lane: DNA template before immunoprecipitation for PCR; 3rd lane: immunoprecipitated DNA template with IgG for negative control. 4th and 5th lanes: immunoprecipitated DNA template with antibodies against SMARCA2 and MeCP2.

and genotype concordance was 99.7%. Genotyping completeness was >0.99.

For a more detailed analysis of the associations of the *SMARCA2* gene, the tag SNPs in the gene were selected using the Haploview program (http://www.broad.mit.edu/mpg/haploview/) with the condition of an r^2 threshold of 0.8 and a minor allele frequency of 0.1, and genotyped by the TaqMan method. Allelic discrimination was performed using the ABI PRISM 7900HT Sequence Detection System using SDS 2.0 software (Applied Biosystems, Foster City, CA, USA).

Cell and animal experiments

Experimental procedures used in cell and animal experiments, including plasmid: construction, knockdown of *SMARCA2* by siRNA, cell culture and transfection, RNA and cDNA preparation, northern and western blot analysis, whole-genome expression analysis, real-time PCR, ChIP assay and behavioral pharmacological analyses of mice are described in the Supplementary Material.

Statistical analysis

Initial screening for association with schizophrenia was done for 11 883 SNPs and, in subsequent analysis, 259 SNPs from the HumanHap370 BeadChip and 34 tag SNPs from *SMARCA2* were added. Therefore, a *P*-value corrected by Bonferroni's method for 12 176 pair-wise comparisons, $P < 4 \times 10^{-6}$, was considered as significant for overall evidence for association. In this study, genotypic *P*-values or haplotype *P*-values were not evaluated to avoid inflation of the *P*-values due to multiple testing.

In the replication study, 5 SNPs from the initial screening, 3 SNPs from genes related to chromatin remodeling and 34 tag SNPs in the *SMARCA2* gene were selected to replicate associations with schizophrenia in the replication samples. A $P = 0.05/(5 + 3 + 34) < 0.001$ was considered as significant in the replication samples. Association and Hardy–Weinberg equilibrium were calculated using chi-test. Haplotype frequencies were estimated using the expectation maximization algorithm. The Haploview program (http://www.broad.mit.edu/mpg/haploview/) was used to detect the haplotype block.

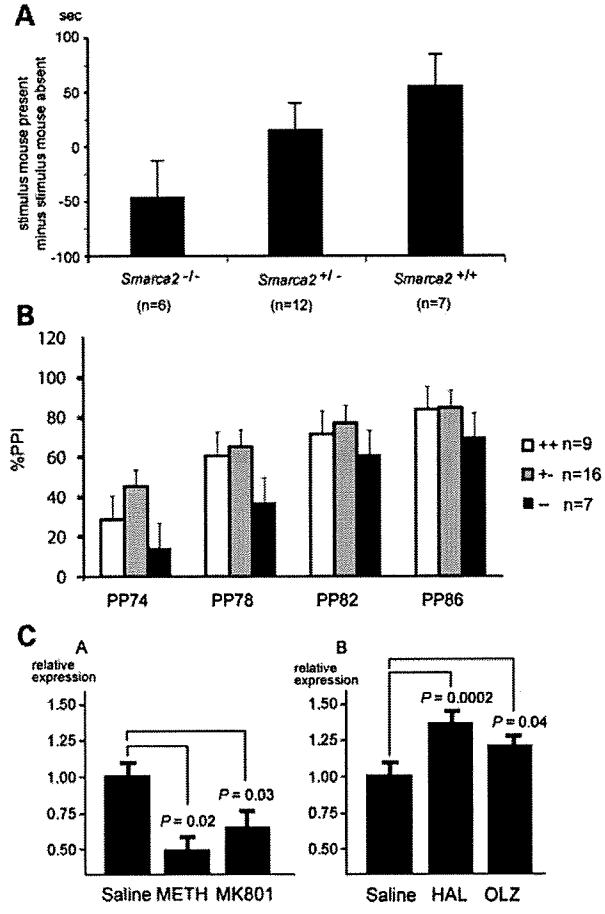

Figure 7. Social interaction and PPI in *Smarca2* −/−, −/+ and +/+ mice and effects of psychotogenic and antipsychotic drugs on *Smarca2* gene expression in the mouse brain. (**A**) The vertical axis is the difference between the time that the test mouse spent sniffing the cylinder where a stimulus mouse was present and the time when stimulus mouse was absent. Genotype was coded as 0, 1 or 2 depending on the number of *Smarca2* copies and a simple regression model was fitted ($P = 0.03$). (**B**) PPI was recorded for *Smarca2* −/−, −/+ and +/+ mice using a conditioning, prepulse noise burst of 74, 78, 82 or 86 dB. *Smarca2* −/− mice had impaired PPI in comparison to heterozygous and wild-type litter-mate mice at 78 dB prepulse noise (F-test, $P = 0.02$), at 82 and 86 dB ($P = 0.07$). (**C**) Effects of psychotogenic and antipsychotic drugs on *Smarca2* gene expression in the mouse brain. *Smarca2* expression levels in the mice brain after treatment with methamphetamine (METH) ($n = 5$), MK-801 ($n = 5$) or saline (control) ($n = 5$) for 12 days (a), haloperidol (HAL) ($n = 10$), olanzapine (OLZ) ($n = 10$) or saline ($n = 10$) for 7 weeks (b). Administration of drugs was by once daily intraperitoneal injection to 4-week-old C57BL/6J male mice. The average relative expression level from the prefrontal cortex, midbrain, hippocampus, thalamus and striatum of the treated group was compared with the saline groups by *t*-test.

In real-time PCR experiments, correlation of SMARCA2 gene expression and diagnosis, ethnicity, age, sex, PMI and the pH of brain samples was analyzed by one-way analysis of variance (ANOVA) tests or regression analyses by JMP computer software version 7. In linear multiple regression analysis, genotypes of the SNPs as qualitative variables, age, sex, PMI, pH, diagnosis and ethnicity of brain samples were included as variables. The genotypes of the SNPs were

assigned to 0, 1 and 2. Differences of *SMARCA2* expression levels between genotypes were analyzed by Student's *t*-tests. A $P < 0.05$ was considered as significant.

SUPPLEMENTARY MATERIAL

Supplementary Material is available at *HMG* online.

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Conflict of Interest statements. Authors declare no conflict of interest.

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Leukemia inhibitory factor gene is associated with schizophrenia and working memory function

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ABSTRACT

Leukemia inhibitory factor (LIF), a member of the interleukin-6 cytokine family, regulates the neuronal phenotype and coordinates astrocyte, oligodendrocyte, microglia, and inflammatory cell responses. The LIF gene is located on 22q12.1–q12.2, a hot spot for schizophrenia. Three polymorphisms of the LIF gene (rs929271, rs737812, and rs929273) were examined in a case–control association study of 390 patients with schizophrenia and 410 age- and sex-matched controls. Effects of a risk genotype of LIF on cognitive domains were evaluated by the Wechsler Adult Intelligence Scale–Revised, Wechsler Memory Scale–Revised, and Wisconsin Card Sorting Test (WCST) in 355 healthy volunteers. The LIF gene showed significant associations with schizophrenia at rs929271 and a haplotype consisting of rs929271–rs737812. After stratification by subtype of schizophrenia, the hebephrenic, but not paranoid, type was associated with the LIF gene at rs929271 (allele, $P=0.014$) and the haplotype (permutation $P=0.013$). Having the T-allele and T-carrier genotypes (TT and TG) of rs929271 were risks for hebephrenic schizophrenia, and the odds ratios were 1.38 (95% CI: 1.21–1.56) and 1.54 (95%CI: 1.19–1.98), respectively. Subjects with T-carrier genotypes made significantly more errors on the WCST compared with those without ($P=0.04$). The present study indicated that the LIF gene variant may produce susceptibility to hebephrenic schizophrenia and deterioration of working memory function.

1. Introduction

Schizophrenia is a severe and complex psychiatric disease characterized by disruption of basic perceptual, cognitive, affective, and judgmental processes, with a lifetime risk of about 1%, and the disorder occurs as a result of interaction between genetic and environmental factors, particularly during the embryological development period. Epidemiological research has indicated that maternal exposure to infection during pregnancy, which induce inflammatory cytokines and activate their signaling cascades, is associated with an increased risk of offspring developing schizophrenia (Brown et al., 2004; Brown, 2008). Analysis of cerebral spinal fluid and serum has revealed altered levels of certain cytokines in schizophrenic patients (Akiyama, 1999; Toyooka et al., 2003), and such changes are considered to be involved in regulating brain development and leading to later perceptual and cognitive alterations (Borrell et al., 2002; Fatemi et al., 2008; Meyer et al., 2008; Nawa et al., 2000; Romero et al., 2008; Tohmi et al., 2004; Urakubo et al., 2001).

Leukemia inhibitory factor (LIF) is a member of the interleukin (IL)-6 cytokine family comprising IL-6, oncostatin M, IL-11, ciliary neurotropic factor, and cardiotropin-1; it signals via binding to a heterodimeric glycoprotein 130 (gp130)/LIF receptor (LIFR) complex (Taga and Kishimoto, 1997). Besides classical hematopoietic effects, LIF was found to regulate neuronal phenotype and coordinate astrocyte, oligodendrocyte, microglia, and inflammatory cell responses (Holmberg and Patterson, 2006; Kerr and Patterson; 2004, 2005; Sugiura et al., 2000). LIF signaling activates the Janus kinase–signal transducer and activator of transcription (JAK/STAT) pathways and induces differentiation of neural precursor cells into astrocytes (Barnabe-Heider et al., 2005; Bonni et al., 1997; He et al., 2005). LIF knock-out (KO) mice display reduced astrocyte and microglial activation in the hippocampus (Holmberg and Patterson, 2006), and LIF-treated rats display increased glial fibrillary acidic protein (GFAP) immunoreactivity, a cytoskeletal marker of astrocyte, in the neocortex (Watanabe et al., 2004). The LIF knock-out mice displayed abnormal prepulse inhibition (PPI) in the acoustic startle test during and after adolescence, which is considered as one of the intermediate phenotypes observed in schizophrenic patients (Watanabe et al., 2004).

Abbreviations: IL, interleukin; LIF, leukemia inhibitory factor; LD, linkage disequilibrium; SNP, single nucleotide polymorphism; WAIS-R, Wechsler Adult Intelligence Scale–Revised; WCST, Wisconsin Card Sorting Test; WMS-R, Wechsler Memory Scale–Revised.

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A proteomic study reported that the levels of GFAP expression reduced in the brains of individuals of schizophrenia (Johnston-Wilson et al., 2000) and another group demonstrated that the GFAP mRNA levels in the white matter of the anterior cingulate cortex decreased in schizophrenic patients (Webster et al., 2005). Serum LIFR concentrations were significantly increased after starting treatment with antipsychotics in schizophrenic patients (Maes et al., 2002). These results suggested that astrocyte has an important role in the pathogenesis of schizophrenia. The LIF gene is located on 22q12.1–q12.2 (Sutherland et al., 1989), which has been implicated as a major mental illness susceptibility locus (Gill et al., 1996; Pulver et al., 1994). Meta-analysis of linkage studies has confirmed that 22q12 has the strongest linkage to schizophrenia (Badner and Gershon, 2002; Lewis et al., 2003; Segurado et al., 2003). Therefore, the LIF gene is one of the candidate genes for association with schizophrenia.

Here we performed, for the first time, the association between the LIF gene and patients with schizophrenia in a Japanese population. To determine the physiological roles of LIF, the association of the LIF gene with memory and intelligence tests in healthy subjects was also examined.

2. Methods

2.1. Subjects

The subjects of the association study comprised 410 unrelated patients fulfilling the ICD-10 (International Classification of Diseases, version 10, WHO 1992) diagnostic criteria for schizophrenia (207 males and 203 females, average age 50.9 ± 13.1 years; 184 were diagnosed with the paranoid subtype, and 199 with the hebephrenic subtype), and 389 age-, sex-, and geographical origin-matched control subjects (187 males and 202 females, average age 50.9 ± 13.9 years). Diagnosis of schizophrenia and determination of subtype and assessment of normal controls were performed by two trained psychiatrists on the basis of all available information and unstructured interviews. Control subjects had no past or family history of central nervous system disease, severe head injury, substance dependence, or mental retardation. All subjects were Japanese. This study was initiated after receiving the approval of the ethical committee of Okayama University Graduate School of Medicine, Dentistry and Pharmaceutical Sciences and the National Center of Neurology and Psychiatry. Written informed consent was obtained from all participants.

2.2. Genotyping

Peripheral blood was obtained from the subjects, and genomic DNA was extracted from peripheral leukocytes using a standard procedure. We selected three single nucleotide polymorphisms (SNPs), rs929271, rs737812, and rs929273, of the LIF gene for genetic association analyses for the following reasons. There were allele frequency data on only 6 SNPs of the LIF gene and the flanking regions when the project was started, and 4 of them showed polymorphism in a Japanese population. Further, the three SNPs showed 10% or higher frequency as minor alleles. Genotyping was performed by TaqMan technology on an ABI7500 Real Time PCR system (Applied Biosystems, U.S.A., rs929271; C_7545904_10, rs737812; C_2624326_1, rs929273; C_2624327_10). Some samples were also analyzed by the restriction fragment length polymorphism method to confirm genotyping (rs929271; *Hinf*I, rs737812; *Hinf*I with mismatch primer, rs929273; *Bsh1236*I with mismatch primer).

2.3. Neuropsychological test measures

Another set of 355 healthy controls (94 males and 261 females, average age 40.6 ± 14.7 years) were subjected to memory and intelligence tests to detect a possible association with the LIF genotype. These individuals were all screened by the Mini-International Neuropsychiatric Interview (Otsubo et al., 2005) with respect to their psychiatric history, and were confirmed to have no current or past history of psychiatric illness. To assess memory and intelligence, the full Japanese versions of the Wechsler Memory Scale–Revised (WMS-R), Wechsler Adult Intelligence Scale–Revised (WAIS-R), and Wisconsin Card Sorting Test (WCST, a modified and computerized version (Kashima et al., 1987; Kobayashi, 1999) were administered. We used a modified and computerized version of the test, and scoring was performed by psychologists who were blind to genotypic data.

2.4. Statistical analysis

Deviation from Hardy–Weinberg equilibrium and the case–control association were examined by χ^2 test. We evaluated pairwise linkage disequilibrium (LD) among the SNPs by χ^2 test, D' value, and r^2. In haplotype analyses, we calculated the permutation P value by using 100,000 simulations to avoid the possibility that a large error could occur in the χ^2 test when the haplotype frequency was extremely small. These statistical analyses were performed by using the software SNPAlyze (Dynacom Co., Japan). The association of the LIF genotype with memory and intelligence was examined by multiple analysis of variance (MANOVA) controlling for possible confounders (age, sex, and years of education) using the SPSS software version 11 (SPSS Japan, Tokyo, Japan). The statistical significance level was set as 0.05.

2.5. GenBank/EMBL accession numbers

Genome: NC_000022.9, NG_008721.1; MIM: 159540.

3. Results

3.1. Genetic association analyses

The genotype distribution and allele frequencies for each polymorphism of patients with schizophrenia and control subjects are shown in Table 1. The genotype distribution of these SNPs did not deviate significantly from Hardy–Weinberg equilibrium. We found a significant association between schizophrenia and allele frequency of rs929271, but not rs737812 and rs929273 of the LIF gene ($\chi^2 = 4.18$, df = 1, $P = 0.041$). As for the subcategories of schizophrenia, hebephrenic type schizophrenia was significantly different from controls in genotype and allele distributions at rs929271 (genotype, $\chi^2 = 6.47$, df = 2, $P = 0.039$; allele, $\chi^2 = 6.26$, df = 1, $P = 0.014$), and in allele distribution at rs929273 ($\chi^2 = 5.55$, df = 1, $P = 0.022$), but the paranoid type did not differ at any SNP. The odds ratios of T-allele and T-carrier genotypes (T/T and T/G) of rs929271 for hebephrenic schizophrenia were 1.38 (95% CI; 1.21–1.56) and 1.54 (1.19–1.98), respectively.

3.2. Haplotype association analysis

Estimation of the pairwise LD between the three SNPs of the LIF gene using the D' and r^2 values as an index revealed that rs929271, rs737812, and rs929273 showed a strong LD (D' ranging 0.98 and 0.99) with each other (Table 2). We then analyzed the haplotype distribution and found significant differences between all patients with schizophrenia and control subjects at a two-loci haplotype that consisted of rs929271–rs727812 (global permutation $P = 0.031$), and between hebephrenic schizophrenia and controls at a two-loci haplotype that consisted of rs929271–rs737812 (global permutation $P = 0.013$) and rs737812–rs929273 (global permutation $P = 0.024$), and a three-loci haplotype that consisted of the three SNPs (global permutation $P = 0.026$, Table 3). The estimated frequency of the haplotype consisting of rs929271–rs737812, which showed the smallest global P value, showed that the T–C frequency was significantly higher in patients with hebephrenic schizophrenia than in control subjects ($P = 0.004$, Table 4). Conversely,

Table 1
Genotype and allele distribution of three single nucleotide polymorphisms of the LIF gene in control subjects and patients with schizophrenia.

	Genotype				Allele		
rs929271	N	T/T	T/G	G/G	P	T	G	P
Control	389	125(32.1)	196(50.4)	68(17.5)		446(57.3)	332(42.7)	
Schizophrenia	409	165(40.3)	180(44.0)	64(15.7)	0.054	510(62.3)	308(37.7)	0.041
Paranoid type	184	76(41.3)	76(41.3)	32(17.4)	0.08	228(62.0)	140(38.0)	0.14
Hebephrenic type	198	83(41.9)	91(46.0)	24(12.1)	0.039	257(64.9)	139(35.1)	0.014
rs737812	N	C/C	C/A	A/A		C	A	
Control	390	291(74.6)	90(23.1)	9(2.3)		672(86.2)	108(13.8)	
Schizophrenia	407	314(75.9)	84(21.8)	9(2.3)	0.70	712(87.5)	102(12.5)	0.44
Paranoid type	183	142(77.6)	37(20.2)	4(2.2)	0.74	321(87.7)	45(12.3)	0.47
Hebephrenic type	198	153(77.3)	40(20.2)	5(2.5)	0.73	346(87.4)	50(12.6)	0.56
rs929273	N	G/G	G/A	A/A		G	A	
Control	389	128(32.9)	193(49.6)	68(17.5)		449(57.7)	329(42.3)	
Schizophrenia	410	164(40.0)	182(44.4)	64(15.6)	0.11	510(62.2)	310(37.8)	0.67
Paranoid type	184	76(41.3)	75(40.8)	33(17.9)	0.10	227(61.7)	141(38.3)	0.20
Hebephrenic type	199	82(41.2)	94(47.2)	23(11.6)	0.06	258(64.8)	140(35.2)	0.02

Numbers in parentheses indicate percentages.

the G–C haplotype was significantly lower in patients than controls ($P=0.011$).

3.3. Association with cognitive function

Because rs929271 showed a significant association with schizophrenia of all patients and the subgroup of hebephrenic patients, we analyzed the effect of this polymorphism on cognitive function using the WMS-R, WAIS-R, and WCST (Table 5). There was no significant difference among the genotype of rs929271 for any sub-scale of the WMS-R, verbal memory, visual memory, general memory, attention/concentration, or delayed recall, or any sub-scale of the WAIS-R, verbal IQ, performance IQ, or full scale IQ. On the other hand, we found significant differences in WCST total errors between those who carried a T allele (T/T and T/G genotypes) and those who did not (G/G). Among subjects with the T-carrier genotype, the T allele was a risk for schizophrenia, and these subjects showed excess errors during the WCST compared to those without the T-carrier genotype.

4. Discussion

The present study revealed that the LIF gene was significantly associated with susceptibility to schizophrenia, especially the hebephrenic type. Thus, the frequencies of T-allele and T-carrier genotypes of rs929271 of the LIF gene were higher in hebephrenic, but not paranoid, schizophrenia patients than in controls, and the odds ratios were 1.38 and 1.54, respectively. The haplotype consisting of rs929271–rs737812 showed that T–C was a significant risk factor and G–C was a protective factor for hebephrenic schizophrenia. In addition, healthy subjects with T-carrier genotypes showed a significant excess of perseveration errors on the WCST compared with non-T carriers, but they showed no difference in other neurocognitive tests, WAIS-R and WMS-R, indicating that LIF may be involved in the working memory measured by the WCST but not in other types of cognitive functions, such as verbal memory, visual memory, attention, delayed recall, and IQ.

Several lines of *in vitro* and *in vivo* evidence support the hypothesis that an imbalance in the fetal brain between pro-inflammatory cytokine signals such as IL-1beta, IL-6, and tumor necrosis factor-alpha and anti-inflammatory signals such as IL-10 may critically affect development of the brain and behaviors in later life, thereby increasing the risk of schizophrenia (Meyer et al., 2008). LIF belongs to the IL-6 cytokine family and mediates inflammatory responses not only in the peripheral tissues but also in the brain by modulating the differentiation of neuronal precursor cells into astrocytes (Barnabe-Heider et al., 2005; Bonni et al., 1997; He et al., 2005). Several studies have revealed that glial cells, particularly astrocytes, are involved in synaptic plasticity by regulating postsynaptic AMPA receptor density and synaptic plasticity underlies higher brain functions such as learning and memory (Bains and Oliet, 2007). A loss of astrocytes or compromised astrocytic function in several cortices has been reported repeatedly in schizophrenic brains (Benes et al., 1986; Stark et al., 2004; Steffek et al., 2008).

The hebephrenic type of schizophrenia is characterized by predominant negative symptoms and disorganization. Schizophrenic patients who had predominantly negative symptoms showed more cognitive impairments than those individuals with schizophrenia with predominantly positive symptoms (Crow, 1980) and a number of studies demonstrated that these negative symptoms were associated with impairments of dorsolateral prefrontal cortex (Sanfilipo et al., 2000; Wolkin et al., 2003). Performance on the WCST may be regarded as a neuropsychological marker of working memory efficiency, depending on the activity of the dorsolateral prefrontal cortex (Weinberger et al., 1986). Previous studies showed that schizophrenic patients showed deficits on WCST performance (Franke et al., 1992; Everett et al., 2001; Martino et al., 2007) and such deficits

Table 2
Pairwise linkage disequilibrium between single nucleotide polymorphisms of LIF gene.

	rs929271	rs737812	rs929273
rs929271		0.98	0.99
rs737812	0.10		0.98
rs929273	0.98	0.10	

Right upper and left lower diagonal showed D' and r^2 values, respectively.

Table 3
Multi-loci association analyses of LIF gene in hebephrenic schizophrenia.

SNP ID	1 locus	2 loci	3 loci
rs929271	0.014		
		0.013	
rs737812	0.56		0.026
		0.024	
rs929273	0.02		