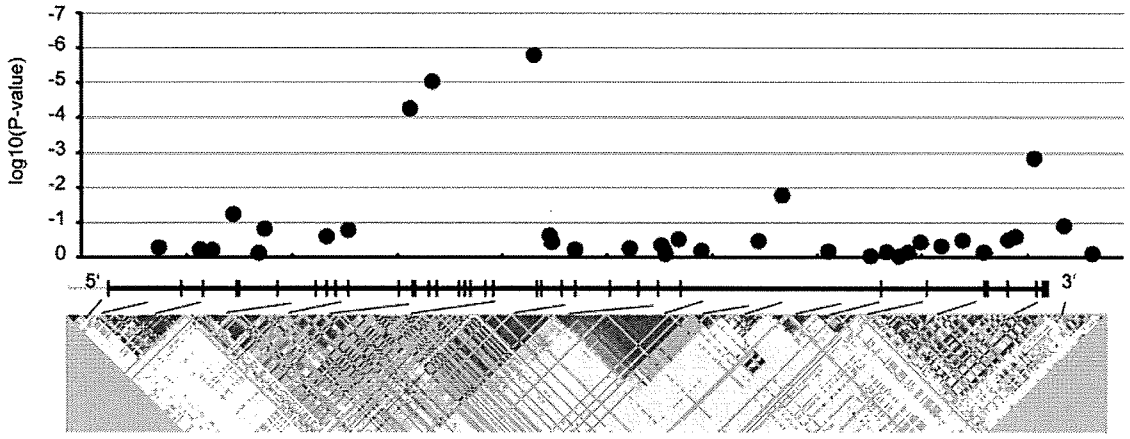

Figure 1. Association of tag SNPs in *SMARCA2* with schizophrenia in the replication samples. Linkage disequilibrium in the HapMap data is also shown with red indicating high linkage disequilibrium and white denoting low linkage disequilibrium. Exons are shown in the middle.

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

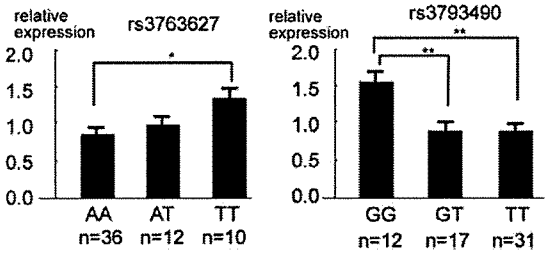

Figure 2. *SMARCA2* expression levels in the postmortem prefrontal region by genotype [rs3763627 (intron 12) and rs3793490 (intron 19)]. The symbols * and ** indicate $P < 0.05$ and $P < 0.01$, respectively, by Student's *t*-test. The A allele of rs3763627 ($P = 0.005$) and the T allele of rs3793490 ($P = 0.01$) 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

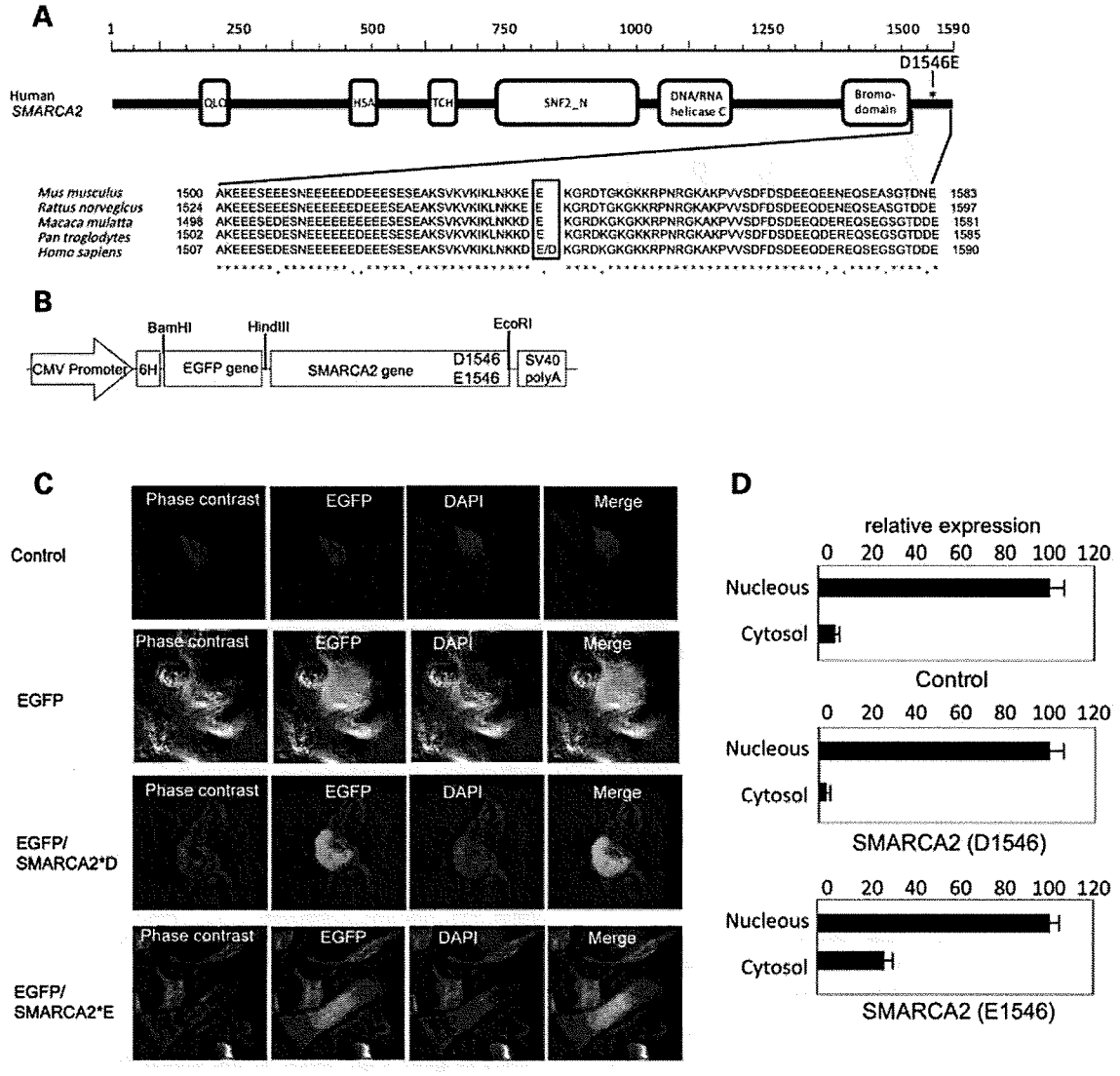

Figure 3. Nuclear localization efficiency of BRM between alleles of rs2296212 (D1546E). (**A**) Domains in BRM and evolutional comparisons of sequences around human D1546E. QLQ, Gln-Leu-Gln motif; HAS, Helicase/SANT-associated, DNA binding; TCH, associated with TFs and helicases; DNA/RNA helicase C; 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).

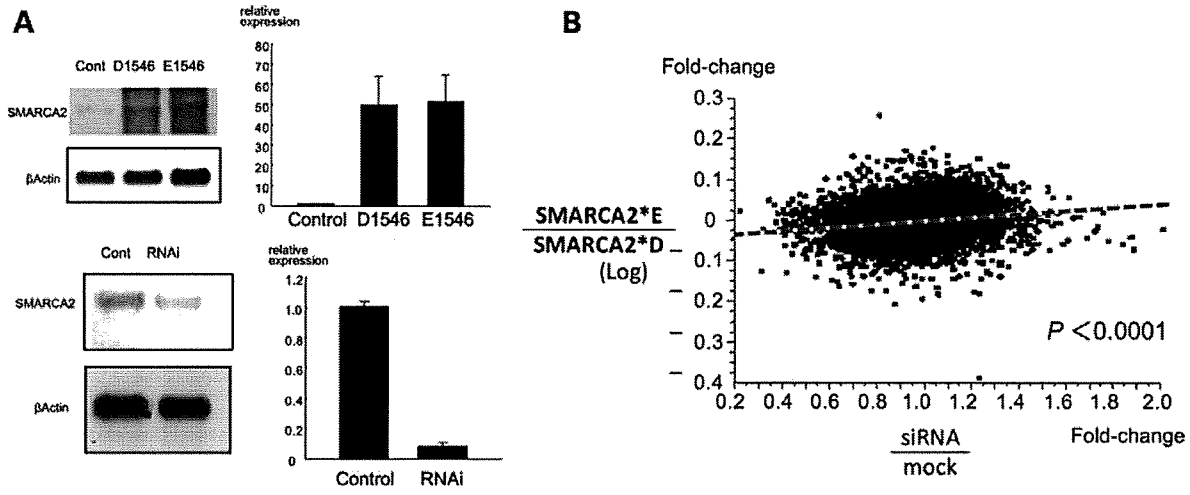

Figure 4. Evaluation of the E1546 and D1546 forms of BRM by gene expression profiles. (**A**) The expression levels of *SMARCA2* in T98G cells transfected by the pSMARCA2*E and *D constructs detected by northern blotting (upper-left) and real-time PCR (upper-right). For real-time PCR, the expression 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

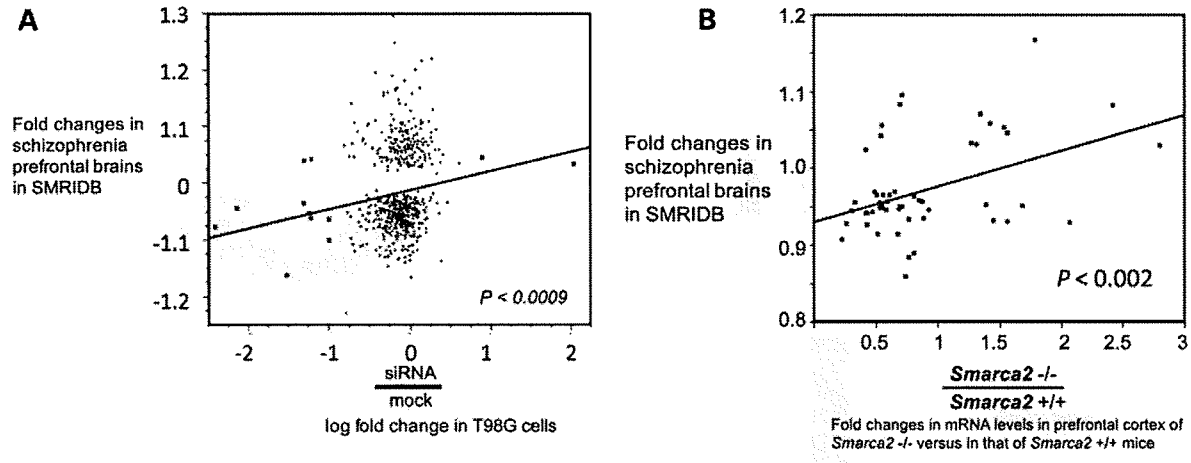

Figure 5. Correlation of transcriptional fold changes in the postmortem prefrontal cortex in SMRIDB with those in T98G cells after siRNA treatments (**A**) and with *Smarca2* knockout prefrontal mouse brains (**B**). Transcriptional fold changes by siRNA against mock in T98G cells were log-transformed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

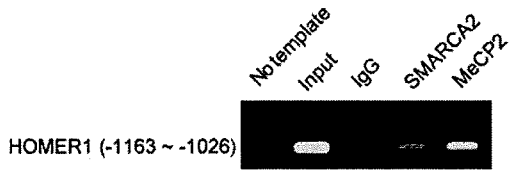

Figure 6. Chromatin immunoprecipitation (ChIP) assays on DNA harvested from T98G cells. DNA that interacts with SMARCA2 and MeCP2 was evaluated using antibodies against to them. Normal rabbit immunoglobulin G (IgG) was used as negative controls. DNA was detected by PCR using primers for the region $-1163 \sim -1036$ 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.

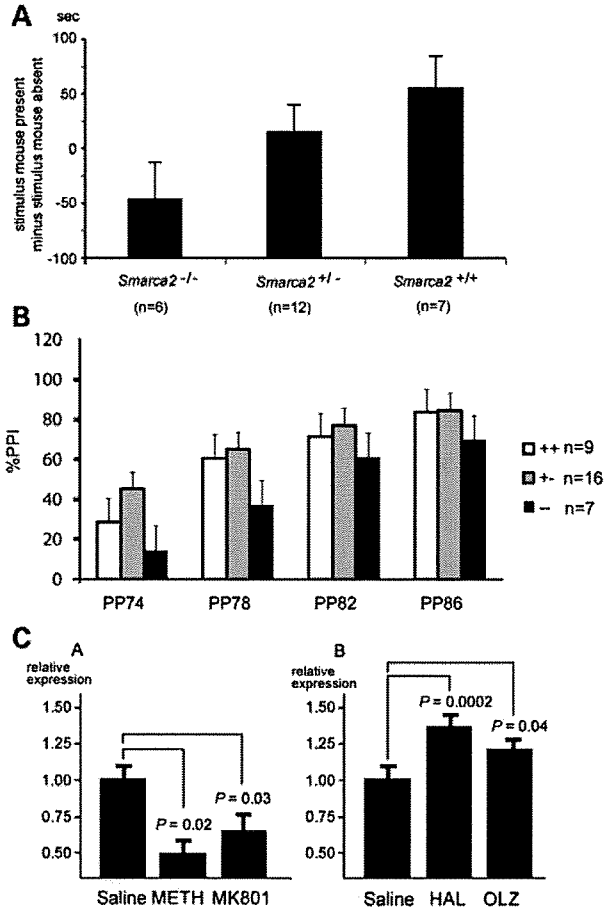

Figure 7. Social interaction and PPI in *Smarca2* −/−, −/+ and +/+ mice and effects of psychotogenic and antipsychotic drugs on *Smarca2* gene expression in the mouse brain. (**A**) The vertical axis is the difference between the time that the test mouse spent sniffing the cylinder where a stimulus mouse 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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REFERENCES

1. Allen, N.C., Bagade, S., McQueen, M.B., Ioannidis, J.P., Kavvoura, F.K., Khoury, M.J., Tanzi, R.E. and Bertram, L. (2008) Systematic meta-analyses and field synopsis of genetic association studies in schizophrenia: the SzGene database. *Nat. Genet.*, **40**, 827–834.
2. Sullivan, P.F., Lin, D., Tzeng, J.Y., van den Oord, E., Perkins, D., Stroup, T.S., Wagner, M., Lee, S., Wright, F.A., Zou, F. *et al.* (2008) Genomewide association for schizophrenia in the CATIE study: results of stage 1. *Mol. Psychiatry*, **13**, 570–584.
3. Lencz, T., Morgan, T.V., Athanasiou, M., Dain, B., Reed, C.R., Kane, J.M., Kucherlapati, R. and Malhotra, A.K. (2007) Converging evidence for a pseudoautosomal cytokine receptor gene locus in schizophrenia. *Mol. Psychiatry*, **12**, 572–580.
4. Kirov, G., Zaharieva, I., Georgieva, L., Moskvina, V., Nikolov, I., Cichon, S., Hillmer, A., Toncheva, D., Owen, M.J. and O'Donovan, M.C. (2008) A genome-wide association study in 574 schizophrenia trios using DNA pooling. *Mol. Psychiatry*, in press.
5. O'Donovan, M.C., Craddock, N., Norton, N., Williams, H., Peirce, T., Moskvina, V., Nikolov, I., Hamshere, M., Carroll, L., Georgieva, L. *et al.* (2008) Identification of loci associated with schizophrenia by genome-wide association and follow-up. *Nat. Genet*, **40**, 1053–1055.
6. Higgs, B.W., Elashoff, M., Richman, S. and Barci, B. (2006) An online database for brain disease research. *BMC Genomics*, **7**, 70.
7. Tsankova, N., Renthal, W., Kumar, A. and Nestler, E.J. (2007) Epigenetic regulation in psychiatric disorders. *Nat. Rev. Neurosci.*, **8**, 355–367.
8. Taniura, H., Sng, J.C. and Yoneda, Y. (2006) Histone modifications in status epilepticus induced by kainate. *Histol. Histopathol.*, **21**, 785–791.
9. Impey, S. (2007) A histone deacetylase regulates addiction. *Neuron*, **56**, 415–417.
10. Schroeder, F.A., Penta, K.L., Matevossian, A., Jones, S.R., Konradi, C., Tapper, A.R. and Akbarian, S. (2008) Drug-induced activation of dopamine D(1) receptor signaling and inhibition of class I/II histone deacetylase induce chromatin remodeling in reward circuitry and modulate cocaine-related behaviors. *Neuropsychopharmacology*, **33**, 2981–2992.
11. Castren, E., Voikar, V. and Rantamaki, T. (2007) Role of neurotrophic factors in depression. *Curr. Opin. Pharmacol.*, **7**, 18–21.
12. Nagarajan, R.P., Hogart, A.R., Gwye, Y., Martin, M.R. and LaSalle, J.M. (2006) Reduced MeCP2 expression is frequent in autism frontal cortex and correlates with aberrant MECP2 promoter methylation. *Epigenetics*, **1**, e1–e11.
13. Bredy, T.W., Wu, H., Crego, C., Zellhoefer, J., Sun, Y.E. and Barad, M. (2007) Histone modifications around individual BDNF gene promoters in prefrontal cortex are associated with extinction of conditioned fear. *Learn. Mem.*, **14**, 268–276.
14. Miller, C.A. and Sweatt, J.D. (2007) Covalent modification of DNA regulates memory formation. *Neuron*, **53**, 857–869.
15. Isles, A.R., Davies, W. and Wilkinson, L.S. (2006) Genomic imprinting and the social brain. *Philos. Trans. R. Soc. Lond. B Biol. Sci.*, **361**, 2229–2237.
16. Fuchikami, M., Morinobu, S., Kurata, A., Yamamoto, S. and Yamawaki, S. (2009) Single immobilization stress differentially alters the expression profile of transcripts of the brain-derived neurotrophic factor (BDNF) gene and histone acetylation at its promoters in the rat hippocampus. *Int. J. Neuropsychopharmacol.*, **12**, 73–82.
17. Weaver, I.C., Cervoni, N., Champagne, F.A., D'Alessio, A.C., Sharma, S., Seckl, J.R., Dymov, S., Szyf, M. and Meaney, M.J. (2004) Epigenetic programming by maternal behavior. *Nat. Neurosci.*, **7**, 847–854.
18. Akbarian, S., Ruehl, M.G., Bliven, E., Luiz, L.A., Peranelli, A.C., Baker, S.P., Roberts, R.C., Bunney, W.E. Jr, Conley, R.C., Jones, E.G. *et al.* (2005) Chromatin alterations associated with down-regulated metabolic gene expression in the prefrontal cortex of subjects with schizophrenia. *Arch. Gen. Psychiatry*, **62**, 829–840.
19. Burns, L.G. and Peterson, C.L. (1997) Protein complexes for remodeling chromatin. *Biochim. Biophys. Acta*, **1350**, 159–168.
20. Carlson, M. and Laurent, B.C. (1994) The SNF/SWI family of global transcriptional activators. *Curr. Opin. Cell Biol.*, **6**, 396–402.
21. Hirschhorn, J.N., Brown, S.A., Clark, C.D. and Winston, F. (1992) Evidence that SNF2/SWI2 and SNF5 activate transcription in yeast by altering chromatin structure. *Genes Dev.*, **6**, 2288–2298.
22. Kingston, R.E., Bunker, C.A. and Imbalzano, A.N. (1996) Repression and activation by multiprotein complexes that alter chromatin structure. *Genes Dev.*, **10**, 905–920.
23. Peterson, C.L. and Herskowitz, I. (1992) Characterization of the yeast SWI1, SWI2 and SWI3 genes, which encode a global activator of transcription. *Cell*, **68**, 573–583.
24. Peterson, C.L. and Workman, J.L. (2000) Promoter targeting and chromatin remodeling by the SWI/SNF complex. *Curr. Opin. Genet. Dev.*, **10**, 187–192.
25. Chiba, H., Muramatsu, M., Nomoto, A. and Kato, H. (1994) Two human homologues of Saccharomyces cerevisiae SWI2/SNF2 and Drosophila brahma are transcriptional coactivators cooperating with the estrogen receptor and the retinoic acid receptor. *Nucleic Acids Res.*, **22**, 1815–1820.
26. Khavari, P.A., Peterson, C.L., Tamkun, J.W., Mendel, D.B. and Crabtree, G.R. (1993) BRG1 contains a conserved domain of the SWI2/SNF2 family necessary for normal mitotic growth and transcription. *Nature*, **366**, 170–174.
27. Muchardt, C. and Yaniv, M. (1993) A human homologue of Saccharomyces cerevisiae SNF2/SWI2 and Drosophila brm genes potentiates transcriptional activation by the glucocorticoid receptor. *EMBO J.*, **12**, 4279–4290.
28. Pazin, M.J. and Kadonaga, J.T. (1997) SWI2/SNF2 and related proteins: ATP-driven motors that disrupt protein-DNA interactions? *Cell*, **88**, 737–740.
29. Schnitzler, G., Sif, S. and Kingston, R.E. (1998) Human SWI/SNF interconverts a nucleosome between its base state and a stable remodeled state. *Cell*, **94**, 17–27.
30. Wang, W., Xue, Y., Zhou, S., Kuo, A., Cairns, B.R. and Crabtree, G.R. (1996) Diversity and specialization of mammalian SWI/SNF complexes. *Genes Dev.*, **10**, 2117–2130.
31. Kadonaga, J.T. (1998) Eukaryotic transcription: an interlaced network of transcription factors and chromatin-modifying machines. *Cell*, **92**, 307–313.
32. Luo, R.X., Postigo, A.A. and Dean, D.C. (1998) Rb interacts with histone deacetylase to repress transcription. *Cell*, **92**, 463–473.
33. Pazin, M.J. and Kadonaga, J.T. (1997) What's up and down with histone deacetylation and transcription? *Cell*, **89**, 325–328.
34. van Lohuizen, M. (1998) Functional analysis of mouse Polycomb group genes. *Cell Mol. Life Sci.*, **54**, 71–79.
35. Wolffe, A.P., Wong, J. and Pruss, D. (1997) Activators and repressors: making use of chromatin to regulate transcription. *Genes Cells*, **2**, 291–302.

36. Harikrishnan, K.N., Chow, M.Z., Baker, E.K., Pal, S., Bassal, S., Brasacchio, D., Wang, L., Craig, J.M., Jones, P.L., Sif, S. *et al.* (2005) Brahma links the SWI/SNF chromatin-remodeling complex with MeCP2-dependent transcriptional silencing. *Nat. Genet.*, **37**, 254–264.
37. Machida, Y., Murai, K., Miyake, K. and Iijima, S. (2001) Expression of chromatin remodeling factors during neural differentiation. *J. Biochem.*, **129**, 43–49.
38. Devlin, B. and Roeder, K. (1999) Genomic control for association studies. *Biometrics*, **55**, 997–1004.
39. Devlin, B., Roeder, K. and Wasserman, L. (2001) Genomic control, a new approach to genetic-based association studies. *Theor. Popul. Biol.*, **60**, 155–166.
40. Tamkun, J.W., Deuring, R., Scott, M.P., Kissinger, M., Pattatucci, A.M., Kaufman, T.C. and Kennison, J.A. (1992) Brahma: a regulator of Drosophila homeotic genes structurally related to the yeast transcriptional activator SNF2/SWI2. *Cell*, **68**, 561–572.
41. Reyes, J.C., Barra, J., Muchardt, C., Camus, A., Babinet, C. and Yaniv, M. (1998) Altered control of cellular proliferation in the absence of mammalian brahma (SNF2alpha). *EMBO J.*, **17**, 6979–6991.
42. Walsh, T., McClellan, J.M., McCarthy, S.E., Addington, A.M., Pierce, S.B., Cooper, G.M., Nord, A.S., Kusenda, M., Malhotra, D., Bhandari, A. *et al.* (2008) Rare structural variants disrupt multiple genes in neurodevelopmental pathways in schizophrenia. *Science*, **320**, 539–543.
43. Numachi, Y., Yoshida, S., Yamashita, M., Fujiyama, K., Naka, M., Matsuoka, H., Sato, M. and Sora, I. (2004) Psychostimulant alters expression of DNA methyltransferase mRNA in the rat brain. *Ann. N. Y. Acad. Sci.*, **1025**, 102–109.
44. Numachi, Y., Shen, H., Yoshida, S., Fujiyama, K., Toda, S., Matsuoka, H., Sora, I. and Sato, M. (2007) Methamphetamine alters expression of DNA methyltransferase 1 mRNA in rat brain. *Neurosci. Lett.*, **414**, 213–217.
45. Li, J., Guo, Y., Schroeder, F.A., Youngs, R.M., Schmidt, T.W., Ferris, C., Konradi, C. and Akbarian, S. (2004) Dopamine D2-like antagonists induce chromatin remodeling in striatal neurons through cyclic AMP-protein kinase A and NMDA receptor signaling. *J. Neurochem.*, **90**, 1117–1131.
46. Brakeman, P.R., Lanahan, A.A., O'Brien, R., Roche, K., Barnes, C.A., Huganir, R.L. and Worley, P.F. (1997) Homer: a protein that selectively binds metabotropic glutamate receptors. *Nature*, **386**, 284–288.
47. Lominac, K.D., Oleson, E.B., Pava, M., Klugmann, M., Schwarz, M.K., Seeburg, P.H., During, M.J., Worley, P.F., Kalivas, P.W. and Szumlinski, K.K. (2005) Distinct roles for different Homer1 isoforms in behaviors and associated prefrontal cortex function. *J. Neurosci.*, **25**, 11586–11594.
48. Szumlinski, K.K., Lominac, K.D., Kleschen, M.J., Oleson, E.B., Dehoff, M.H., Schwarz, M.K., Seeburg, P.H., Worley, P.F. and Kalivas, P.W. (2005) Behavioral and neurochemical phenotyping of Homer1 mutant mice: possible relevance to schizophrenia. *Genes Brain Behav.*, **4**, 273–288.
49. Reisman, D.N., Sciarrotta, J., Wang, W., Funkhouser, W.K. and Weissman, B.E. (2003) Loss of BRG1/BRM in human lung cancer cell lines and primary lung cancers: correlation with poor prognosis. *Cancer Res.*, **63**, 560–566.
50. Yamamichi, N., Inada, K., Ichinose, M., Yamamichi-Nishina, M., Mizutani, T., Watanabe, H., Shiogama, K., Fujishiro, M., Okazaki, T., Yahagi, N. *et al.* (2007) Frequent loss of Brm expression in gastric cancer correlates with histologic features and differentiation state. *Cancer Res.*, **67**, 10727–10735.
51. Sun, A., Tawfik, O., Gayed, B., Thrasher, J.B., Hoestje, S., Li, C. and Li, B. (2007) Aberrant expression of SWI/SNF catalytic subunits BRG1/BRM is associated with tumor development and increased invasiveness in prostate cancers. *Prostate*, **67**, 203–213.
52. Hippisley-Cox, J., Vinogradova, Y., Coupland, C. and Parker, C. (2007) Risk of malignancy in patients with schizophrenia or bipolar disorder: nested case–control study. *Arch. Gen. Psychiatry*, **64**, 1368–1376.
53. Torrey, E.F. (2006) Prostate cancer and schizophrenia. *Urology*, **68**, 1280–1283.
54. Catts, V.S., Catts, S.V., O'Toole, B.I. and Frost, A.D. (2008) Cancer incidence in patients with schizophrenia and their first-degree relatives—a meta-analysis. *Acta Psychiatr. Scand.*, **117**, 323–336.
55. Abecasis, G.R., Cherny, S.S., Cookson, W.O. and Cardon, L.R. (2001) GRR: graphical representation of relationship errors. *Bioinformatics*, **17**, 742–743.
56. Ishiguro, H., Koga, M., Horiuchi, Y., Noguchi, E., Morikawa, M., Suzuki, Y., Arai, M., Niizato, K., Iritani, S., Itokawa, M. *et al.* (2008) Supportive evidence for reduced expression of GNB1L in schizophrenia. *Schizophr. Bull*, in press.

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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		

Table 4
Haplotype frequencies of LIF gene of control subjects and patients with hebephrenic schizophrenia.

Haplotype rs929271–rs737812	Controls Frequency	Patients Frequency	Permutation *P*
T–C	0.4344	0.5254	0.0040
G–C	0.4267	0.3503	0.011
T–A	0.1388	0.1244	–

Haplotype analysis was performed by the permutation method. The global permutation *P* was 0.013.

were also found in healthy first-degree relatives of schizophrenic patients (Rybakowski and Borkowska, 2002). Suhr and Spitznagel (2001) reported that high negative symptoms were associated with poor performance on the WCST. Taking these findings together with ours, it can be speculated that T-carrier genotypes of rs929271 of the LIF gene may alter the pro-inflammatory cytokine response to infections during fetal or early life, which may result in neurocognitive maldevelopment of working memory in later life, which are physiological bases for thought disturbance. Finally, a LIF variant may produce susceptibility to hebephrenic schizophrenia, a prominent feature of which is thought disturbance.

Some points should be considered in the present study. We found a statistically significant association of the LIF gene with schizophrenia, but the association was marginal, and we did not apply a multiple test correction. The possibility of a type I error should be considered. As we examined only three SNPs of the LIF gene, although they cover almost the entire LIF gene, the present study does not exclude the possibility of associations between other variations in the LIF gene and schizophrenia. Further replication studies by examining of additional SNPs in large sample populations are necessary to confirm our findings.

We examined healthy subjects to examine the role of the LIF gene in neurocognition, but not in patients with schizophrenia. In spite of recommendations as to the optimal prescription of antipsychotics, antipsychotic polypharmacy and excessive dosing are still highly prevalent worldwide, especially in Japan (Faries et al., 2005; Procyshyn et al., 2001; Sim et al., 2004a,b). It was suggested that the cognitive deficits of patients with schizophrenia may result from non-standard use, polypharmacy, and overdose of antipsychotics (Hori et al., 2006). On the other hand, some studies reported that the WCST score of schizophrenic patients was improved after administration with atypical antipsychotics (Rybakowski et al., 2007; Gallhofer et al., 2007). We did not examine the association between the LIF gene and cognitive decline in patients because almost all of the patients in the present study were taking antipsychotic medications, and their effects on neurocognitive testing cannot be denied. Further studies including unmedicated patients and a more precise, advanced statistical approach are necessary to understand the effects of the LIF gene on susceptibility to schizophrenia and cognitive function. In addition, examination of association between LIF and other endophenotypes, including prepulse inhibition may be necessary for further understanding the physiological roles of the LIF gene.

In conclusion, the present study indicated that the LIF gene variant may produce susceptibility to hebephrenic schizophrenia and deterioration of working memory function.

Table 5
Results of WCST with genotype of rs929271.

	T carrier	Non-T carrier	*P*
Verbal IQ	109.5 ± 12.3	107.5 ± 12.8	0.36
Performance IQ	110.9 ± 11.3	109.8 ± 12.6	0.52
Verbal memory	111.5 ± 14.0	111.0 ± 14.9	0.48
Visual memory	109.8 ± 9.5	107.5 ± 12.5	0.23
Attention	105.1 ± 13.6	105.6 ± 13.6	0.50
Delayed recall	112.2 ± 12.1	112.8 ± 13.2	0.43
WCST-CA	3.55 ± 2.06	3.82 ± 1.89	0.16
WCST-TE	18.29 ± 8.50	16.62 ± 6.42	0.04

The scores of T-allele carriers (T/T or T/G) of rs929271 and non-carriers (G/G) were compared. WCST-CA, WCST: category achieved; WCST-TE, WCST: total errors.
P values were examined by MANOVA to adjust for age, sex and education years.

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References

Akiyama K. Serum levels of soluble IL-2 receptor alpha, IL-6 and IL-1 receptor antagonist in schizophrenia before and during neuroleptic administration. Schizophr Res 1999;37:97-106.
Badner JA, Gershon ES. Meta-analysis of whole-genome linkage scans of bipolar disorder and schizophrenia. Mol Psychiatry 2002;7:405–11.
Bains JS, Oliet SH. Glia: they make your memories stick! Trends Neurosci 2007;30:417–24.
Barnabe-Heider F, Wasylnka JA, Fernandes KJ, Porsche C, Sendtner M, Kaplan DR, et al. Evidence that embryonic neurons regulate the onset of cortical gliogenesis via cardiotrophin-1. Neuron 2005;48:253–65.
Benes FM, Davidson J, Bird ED. Quantitative cytoarchitectural studies of the cerebral cortex of schizophrenics. Arch Gen Psychiatry 1986;43:31–5.
Bonni A, Sun Y, Nadal-Vicens M, Bhatt A, Frank DA, Rozovsky I, et al. Regulation of gliogenesis in the central nervous system by the JAK–STAT signaling pathway. Science 1997;278:477–83.
Borrell J, Vela JM, Arevalo-Martin A, Molina-Holgado E, Guaza C. Prenatal immune challenge disrupts sensorimotor gating in adult rats. Implications for the etiopathogenesis of schizophrenia. Neuropsychopharmacology 2002;26:204–15.
Brown AS. The risk for schizophrenia from childhood and adult infections. Am J Psychiatry 2008;165:7-10.
Brown AS, Begg MD, Gravenstein S, Schaefer CA, Wyatt RJ, Bresnahan M, et al. Serologic evidence of prenatal influenza in the etiology of schizophrenia. Arch Gen Psychiatry 2004;61:774–80.
Crow TJ. Molecular pathology of schizophrenia: more than one disease process? Br Med J 1980;280:66–8.
Everett J, Lavoie K, Gagnon JF, Gosselin N. Performance of patients with schizophrenia on the Wisconsin Card Sorting Test (WCST). J Psychiatry Neurosci 2001;26:123–30.
Faries D, Ascher-Svanum H, Zhu B, Correll C, Kane J. Antipsychotic monotherapy and polypharmacy in the naturalistic treatment of schizophrenia with atypical antipsychotics. BMC Psychiatry 2005;5:26.
Fatemi SH, Reutiman TJ, Folsom TD, Huang H, Oishi K, Mori S, et al. Maternal infection leads to abnormal gene regulation and brain atrophy in mouse offspring: implications for genesis of neurodevelopmental disorders. Schizophr Res 2008;99:56–70.
Franke P, Maier W, Hain C, Klingler T. Wisconsin Card Sorting Test: an indicator of vulnerability to schizophrenia? Schizophr Res 1992;6:243–9.
Gallhofer B, Jaanson P, Mirttoux A, Tanghoj P, Lis S, Krieger S. Course of recovery of cognitive impairment in patients with schizophrenia: a randomized double-blind study comparing sertindole and haloperidole. Pharmacopsychiatry 2007;40:275–86.
Gill M, Vallada H, Collier D, Sham P, Holmans P, Murray R, et al. A combined analysis of D22S278 marker alleles in affected sib-pairs: support for a susceptibility locus for schizophrenia at chromosome 22q12. Schizophrenia Collaborative Linkage Group (Chromosome 22). Am J Med Genet 1996;67:40–5.
He F, Ge W, Martinowich K, Becker-Catania S, Coskun V, Zhu W, et al. A positive autoregulatory loop of Jak–STAT signaling controls the onset of astrogliogenesis. Nat Neurosci 2005;8:616–25.
Holmberg KH, Patterson PH. Leukemia inhibitory factor is a key regulator of astrocytic, microglial and neuronal responses in a low-dose pilocarpine injury model. Brain Res 2006;1075:26–35.
Hori H, Noguchi H, Hashimoto R, Nakabayashi T, Omori M, Takahashi S, et al. Antipsychotic medication and cognitive function in schizophrenia. Schizophr Res 2006;86:138–46.
Johnston-Wilson NL, Sims CD, Hofmann JP, Anderson L, Shore AD, Torrey EF, et al. Disease-specific alternations in frontal cortex brain proteins in schizophrenia, bipolar disorder, and major depressive disorder. Mol Psychiatry 2000;5:142–9.
Kashima H, Handa T, Kato M, Sakura K, Yokoyama N, Murakami M, et al. Neuropsychological investigation on chronic schizophrenia—aspects of its frontal functions. In: Takahashi R, Flor-Henry P, Gruzelier J and Niwa S, (Eds.), Cerebral Dynamics, Laterality and Psychopathology, Elsevier, Amsterdam 1987, p. 337–45
Kerr BJ, Patterson PH. Potent pro-inflammatory actions of leukemia inhibitory factor in the spinal cord of the adult mouse. Exp Neurol 2004;188:391–407.
Kerr BJ, Patterson PH. Leukemia inhibitory factor promotes oligodendrocyte survival after spinal cord injury. Glia 2005;51:73–9.
Kobayashi S. Wisconsin Card Sorting Test Program Keio-F-S-version (Web site, in Japanese) 1999. Available at:http://cvddb.shimane-med.ac.jp/user/wisconsin.htm
Lewis CM, Levinson DF, Wise LH, DeLisi LE, Straub RE, Hovatta I, et al. Genome scan meta-analysis of schizophrenia and bipolar disorder, part II: schizophrenia. Am J Hum Genet 2003;73:34–48.
Maes M, Bocchio Chiavetto L, Bignotti S, Battisa Tura GJ, Pioli R, Boin F, et al. Increased serum interleukin-8 and interleukin-10 in schizophrenic patients resistant to treatment with neuroleptics and the stimulatory effects of clozapine on serum leukemia inhibitory factor receptor. Schizophr Res 2002;54:281–91.
Martino DJ, Bucay D, Butnam JT, Allegri RF. Neuropsychological frontal impairments and negative symptoms in schizophrenia. Psychiatry Res 2007;59:127–8.

Meyer U, Feldon J, Yee BK. A review of the fetal brain cytokine imbalance hypothesis of schizophrenia. Schizophr Bull 2008;35:959–72.
Nawa H, Takahashi M, Patterson PH. Cytokine and growth factor involvement in schizophrenia—support for the developmental model. Mol Psychiatry 2000;5:594–603.
Otsubo T, Tanaka K, Koda R, Shinoda J, Sano N, Tanaka S, et al. Reliability and validity of Japanese version of the Mini-International Neuropsychiatric Interview. Psychiatry Clin Neurosci 2005;59:517–26.
Procyshyn RM, Kennedy NB, Tse G, Thompson B. Antipsychotic polypharmacy: a survey of discharge prescriptions from a tertiary care psychiatric institution. Can J Psychiatry 2001;46:334–9.
Pulver AE, Karayiorgou M, Wolyniec PS, Lasseter VK, Kasch L, Nestadt G, et al. Sequential strategy to identify a susceptibility gene for schizophrenia: report of potential linkage on chromosome 22q12–q13.1: part 1. Am J Med Genet 1994;54:36–43.
Romero E, Guaza C, Castellano B, Borrell J. Ontogeny of sensorimotor gating and immune impairment induced by prenatal immune challenge in rats: implications for the etiopathology of schizophrenia. Mol Psychiatry 2008; in press. doi:10.1038/m.p.2008.44.
Rybakowski JK, Borkowska A. Eye movement and neuropsychological studies in first-degree relatives of schizophrenic patients. Schizophr Res 2002;54:105–10.
Rybakowski JK, Drozdz W, Borkowska A. Long-term administration of the low-dose risperidone in schizotaxia subjects. Hum Psychopharmacol 2007;22:407–12.
Sanfilipo M, Lafargue T, Rusinek H, Arena L, Loneragan C, Lautin A, et al. Volumetric measures of the frontal and temporal lobe regions in schizophrenia: relationship to negative symptoms. Arch Gen Psychiatry 2000;57:471–80.
Segurado R, Detera-Wadleigh SD, Levinson DF, Lewis CM, Gill M, Nurnberger Jr JI, et al. Genome scan meta-analysis of schizophrenia and bipolar disorder, part III: bipolar disorder. Am J Hum Genet 2003;73:49–62.
Sim K, Su A, Fujii S, Yang SY, Chong MY, Ungvari GS, et al. Antipsychotic polypharmacy in patients with schizophrenia: a multicentre comparative study in East Asia. Br J Clin Pharmacol 2004a;58:178–83.
Sim K, Su A, Leong JY, Yip K, Chong MY, Fujii S, et al. High dose antipsychotic use in schizophrenia: findings of the REAP (research on east Asia psychotropic prescriptions) study. Pharmacopsychiatry 2004b;37:175–9.
Stark AK, Uylings HB, Sanz-Arigita E, Pakkenberg B. Glial cell loss in the anterior cingulate cortex, a subregion of the prefrontal cortex, in subjects with schizophrenia. Am J Psychiatry 2004;161:882–8.
Steffek AE, McCullumsmith RE, Haroutunian V, Meador-Woodruff JH. Cortical expression of glial fibrillary acidic protein and glutamine synthetase is decreased in schizophrenia. Schizophr Res 2008;103:71–82.
Sugiura S, Lahav R, Han J, Kou SY, Banner LR, de Pablo F, et al. Leukaemia inhibitory factor is required for normal inflammatory responses to injury in the peripheral and central nervous systems in vivo and is chemotactic for macrophages in vitro. Eur J Neurosci 2000;12:457–66.
Suhr JA, Spitznagel MB. Factor versus cluster models of schizotypal traits. II: relation to neuropsychological impairment. Schizophr Res 2001;52:241–50.
Sutherland GR, Baker E, Hyland VJ, Callen DF, Stahl J, Gough NM. The gene for human leukemia inhibitory factor (LIF) maps to 22q12. Leukemia 1989;3:9-13.
Taga T, Kishimoto T. Gp130 and the interleukin-6 family of cytokines. Annu Rev Immunol 1997;15:797–819.
Tohmi M, Tsuda N, Watanabe Y, Kakita A, Nawa H. Perinatal inflammatory cytokine challenge results in distinct neurobehavioral alterations in rats: implication in psychiatric disorders of developmental origin. Neuro Res 2004;50:67–75.
Toyooka K, Watanabe Y, Iritani S, Shimizu E, Iyo M, Nakamura R, et al. A decrease in interleukin-1 receptor antagonist expression in the prefrontal cortex of schizophrenic patients. Neurosci Res 2003;46:299–307.
Urakubo A, Jarskog LF, Lieberman JA, Gilmore JH. Prenatal exposure to maternal infection alters cytokine expression in the placenta, amniotic fluid, and fetal brain. Schizophr Res 2001;47:27–36.
Watanabe Y, Hashimoto S, Kakita A, Takahashi H, Ko J, Mizuno M, et al. Neonatal impact of leukemia inhibitory factor on neurobehavioral development in rats. Neurosci Res 2004;48:345–53.
Webster MJ, O'Grady J, Kleinman JE, Weickert CS. Glial fibrillary acidic protein mRNA levels in the cingulated cortex of individuals with depression, bipolar disorder and schizophrenia. Neuroscience 2005;133:453–61.
Weinberger DR, Berman KF, Zec RF. Physiological dysfunction of dorsolateral prefrontal cortex in schizophrenia. I. Regional cerebral blood flow evidence. Arch Gen Psychiatry 1986;43:114–24.
Wolkin A, Choi SJ, Szilagyi S, Sanfilipo M, Rotrosen JP, Lim KO. Inferior frontal white matter anisotropy and negative symptoms of schizophrenia: a diffusion tensor imaging study. Am J Psychiatry 2003;160:572–4.

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Possible association of the semaphorin 3D gene (*SEMA3D*) with schizophrenia

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ABSTRACT

Semaphorins are ligands of plexins, and the plexin–semaphorin signaling system is widely involved in many neuronal events including axon guidance, cell migration, axon pruning, and synaptic plasticity. The plexin A2 gene (*PLXNA2*) has been reported to be associated with schizophrenia. This finding prompted us to examine the possible association between the semaphorin 3D gene (*SEMA3D*) and schizophrenia in a Japanese population. We genotyped 9 tagging single nucleotide polymorphisms (SNPs) of *SEMA3D* including a non-synonymous variation, Lys701Gln (rs7800072), in a sample of 506 patients with schizophrenia and 941 healthy control subjects. The Gln701 allele showed a significant protective effect against the development of schizophrenia ($p = 0.0069$, odds ratio = 0.76, 95% confidence interval 0.63 to 0.93). Furthermore, the haplotype-based analyses revealed a significant association. The four-marker analysis (rs2190208–rs1029564–rs17159614–rs12176601), in particular, not including the Lys701Gln, revealed a highly significant association ($p = 0.00001$, global permutation), suggesting that there may be other functional polymorphisms within *SEMA3D*. Our findings provide strong evidence that *SEMA3D* confers susceptibility to schizophrenia, which could contribute to the neurodevelopmental impairments in the disorder.

1. Introduction

The first discovered semaphorin, collapsing-1 (now Sema3A), was originally reported as a repulsive cue in axon guidance (Luo et al., 1993). To date, more than 20 semaphorins of secreted or membrane forms have been identified in various species ranging from nematodes to humans (Luo et al., 1993; Fujii et al., 2002; Yazdani and Terman, 2006). Semaphorins act as ligands for plexins, and the plexin–semaphorin signaling system has been widely investigated in nervous systems (Mann et al., 2007). Class 3 semaphorins (SEMA3A-G) have been well-studied and generally act as secreted ligands for the heterodimerized complex of the plexin A family members and neuropilins (Fujisawa, 2004). For example, Sema3A binds to neuropilin-1 and activates plexin A1 or plexin A2 to transduce a repulsive axon guidance signal (Takahashi and Strittmatter, 2001). Many studies of the plexin–semaphorin signaling system have concentrated on their roles in neuronal development and plasticity (reviewed in (Kruger et al., 2005; Halloran and Wolman, 2006; Waimey and Cheng, 2006; Mann et al., 2007)). Q1

Recently, the relationship between schizophrenia and molecules in the plexin–semaphorin signaling system has begun to receive much attention, for several reasons (Mann et al., 2007). An increase in levels of SEMA3A was noted in the cerebellum in postmortem brains of schizophrenia patients, as measured by immunoreactivity in the inner molecular layer and by the enzyme-linked immunosorbent assay (ELISA) in cerebellar protein extract (Eastwood et al., 2003). A genome-wide association study using 25,494 single nucleotide polymorphisms (SNPs) revealed that an intronic SNP of *PLXNA2* was most consistently associated with schizophrenia in European–American populations (Mah et al., 2006). Our replication study in a Japanese sample failed to confirm such an association (Fujii et al., 2007); however, a meta-analysis combining data from previous studies of *PLXNA2* yielded a positive association with schizophrenia (Allen et al., 2008), in which it was reported that the C allele of the SNP rs752016 of *PLXNA2* showed a nominally significant protective effect (odds ratios (OR) = 0.82, 95%

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confidence interval (CI) = 0.69–0.99), and association of the SNP rs841865 approached statistical significance (OR = 0.84, 95% CI = 0.69–1.01) when samples of Mah et al. and Fujii et al. were combined (Mah et al., 2006; Fujii et al., 2007). Furthermore, in the updated online database, "SchizophreniaGene (http://www.schizophreniaforum.org/)," association of the SNP rs1327175 approached statistical significance (OR = 0.76, 95% CI = 0.57–1.00) (Mah et al., 2006; Fujii et al., 2007; Takeshita et al., 2008; Budel et al., 2008). Therefore, genes of the plexin family, the semaphorin family, and neuropilins, are intriguing candidates for schizophrenia susceptibility genes. We then focused on *SEMA3D* as a candidate gene for schizophrenia. *SEMA3D* was mapped to chromosome 7q21 (Clark et al., 2003); interestingly, a previous genome-wide scan suggested that this chromosomal region contains a susceptibility locus for schizophrenia (Ekelund et al., 2000) and recent studies have provided additional support for this possibility (Tastemir et al., 2006; Wedenoja et al., 2008, 2009; Idol et al., 2008).

The aim of the present study was to examine the possible association between *SEMA3D* and schizophrenia. *SEMA3D* has a common variant in the coding region due to an A to C base substitution (rs7800072), which results in an amino acid change (701 Lys to Gln). This SNP has previously been examined with regard to brain morphology (assessed with magnetic resonance imaging) in patients with schizophrenia (Gregorio et al., 2009). Although this study failed to find significant alterations in brain morphology, it is still unclear whether this SNP confers susceptibility to schizophrenia. We examined the possible association of schizophrenia with this non-synonymous SNP, plus 8 tagging SNPs encompassing the entire *SEMA3D* gene.

2. Subjects and methods

2.1. Subjects

Subjects were 506 patients with schizophrenia (278 males [54.9%], mean age 44.3 years [SD 14.1]) and 941 healthy controls (334 males [35.5%], mean age 44.8 years [SD 16.3]). All subjects were Japanese, biologically unrelated, and recruited from the same geographical area (Western part of Tokyo Metropolitan). Consensus diagnosis by at least two psychiatrists was made for each patient according to the Diagnostic and Statistical Manual of Mental Disorders, 4th edition (DSM-IV) criteria (American Psychiatric Association, 1994) on the basis of unstructured interviews and information from medical records. The controls were healthy volunteers recruited from the same geographical area. Control individuals were interviewed and those who had a current or past history of psychiatric treatment were not enrolled in the study. The study protocol was approved by the ethics committee of the National Center of Neurology and Psychiatry, Japan. After description of the study, written informed consent was obtained from every subject.

2.2. SNP selection

The tagging SNPs were selected using the phase III version of HapMap (http://www.hapmap.org/cgi-perl/gbrowse/). SNP genotype data for the JPT (Japanese in Tokyo, Japan) were downloaded for the genomic region of *SEMA3D* plus 2 kb 5′ and 2 kb 3′ of this region (chr7q21.11). The most centromeric and telomeric HapMap markers downloaded were rs6944966 and rs11762367, respectively. HapMap markers were analyzed using the Haploview 4.1 system (http://www.broad.mit.edu/mpg/haploview) with the following criteria of marker selection: Hardy–Weinberg (HW) p value cutoff: 0.05; minimum genotypes: 90%; maximum number of Mendelian errors: 1; minimum minor allele frequency: 0.1; minimum distance between tags: 10 kb. Tagging SNPs were selected using the Tagger function implemented in Haploview with the following criteria: pairwise tagging only and r^2 threshold 0.8. We preselected rs7800072 and rs6966472 as markers and used the Tagger function implemented in Haploview to select other markers. As a result, 9 markers were selected as suitable for analysis for *SEMA3D*. SNP rs7800072 is non-synonymous (2141A > C, Lys701Gln). The numbers of base and amino acid positions were according to NM_152754.2 and NP_689967.2, respectively.

2.3. Genotyping

Venous blood was drawn from the subjects and genomic DNA was extracted from whole blood according to standard procedures. The SNPs were genotyped using the TaqMan 5′-exonuclease allelic discrimination assay; the assay ID (Applied Biosystems, Foster City, CA) of each SNP was C__15937080_10 for rs2190208, C___7585979_10 for rs1029564, C__33462384_10 for rs17159614, C__31373903_10 for rs12176601, C___2635874_10 for rs6966472, C___2635864_10 for rs17559978, C__33462432_10 for rs17159577, C__33462438_10 for rs17159556, and C__25994972_10 for rs7800072. Thermal cycling conditions for polymerase chain reaction (PCR) were 1 cycle at 95 °C for 10 min followed by 50 cycles of 92 °C for 15 s and 60 °C for 1 min. Genotype data were read blind to the case-control status. Ambiguous genotype data were not included in the analysis.

2.4. Haplotype and statistical analysis

Deviations of genotype distributions from the HW equilibrium (HWE) were assessed with the χ^2 test for goodness of fit. Genotype and allele distributions were compared between patients and controls by using the χ^2 test for independence. These tests were performed with SPSS software ver.11 (SPSS Japan, Tokyo, Japan). Haplotype-based association analyses were performed with SNPAlyze software ver.6.5 (http://www.dynacom.co.jp/e/products/package/snpalyze/about.html). The measures of linkage disequilibrium (LD), denoted as D′ and r^2, were calculated from the haplotype frequency using the expectation-maximization (EM) algorithm. Haplotypes with frequencies of less than 1% were considered to be rare and were excluded from the analyses. All p values reported are two-tailed. We performed 100,000 permutations only for some significant haplotypes (e.g., rs2190208–rs1029564–rs17159614–rs121176601) and 10,000 permutations for the other haplotypes. OR and 95% CI were also calculated. To correct the critical p value for multiple testing, we used the spectral decomposition method of SNPSpD software (http://gump.qimr.edu.au/general/daleN/SNPSpD/) (Nyholt, 2004; Li and Ji, 2005), which considers marker linkage disequilibrium information and generates an experiment-wide significance threshold required to keep the type I error rate at 5%.

3. Results

Genotype and allele distributions of the examined SNPs of *SEMA3D* in patients and controls are shown in Table 1. LD estimates of pairwise SNPs, expressed in D′ and r^2, are presented in Fig. 1. The genotype distributions did not significantly deviate from the HWE in patients and controls for any of the examined SNPs. For the non-synonymous polymorphism of *SEMA3D* (rs7800072), there were significant differences in both genotype ($\chi^2 = 8.7$, df = 2, $p = 0.013$) and allele ($\chi^2 = 7.3$, df = 1, $p = 0.0069$, OR = 0.76, 95% CI 0.63–0.93) distributions between patients and controls (Table 1). Furthermore, with respect to the other 8 SNPs (rs2190208, rs1029564,

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Table 1
Genotype and Allelic Distribution of the SEMA3D SNPs in Japanese Patients with Schizophrenia, and Controls.

dbSNP ID	position[a]	Inter-SNP distance (bp)	Group	N	Genotype distribution (frequency)			Allele distribution (frequency)		Odds ratio (95% CI)	Chi-square test[b]		
											HWE(df = 1)[c]	GF(df = 2)[d]	AF(df = 1)[e]
rs2190208	9986227				GG	GA	AA	G	A				
	5′ promoter	——	Schizophrenia	494	186 (0.38)	231 (0.47)	77 (0.16)	603 (0.61)	385 (0.39)	0.96	$\chi2 = 0.14$, $p = 0.71$	$p = 0.48$	$p = 0.59$
			Control	930	325 (0.35)	466 (0.50)	139 (0.15)	1116 (0.60)	744 (0.40)	(0.82–1.12)	$\chi2 = 1.79$, $p = 0.18$	$\chi2 = 1.48$	$\chi2 = 0.29$
rs10295564	9974131				AA	AC	CC	A	C				
	intron 1	12096	Schizophrenia	492	334(0.68)	140 (0.28)	18 (0.04)	808 (0.82)	176 (0.18)	0.78	$\chi2 = 0.48$, $p = 0.48$	$\mathbf{p = 0.028}$	$\mathbf{p = 0.011}$
			Control	931	565 (0.61)	324 (0.35)	42 (0.05)	1454 (0.78)	408 (0.22)	(0.64–0.94)	$\chi2 = 0.27$, $p = 0.61$	$\chi2 = 7.17$	$\chi2 = 6.40$
rs17159614	9959778				GG	GA	AA	G	A				
	intron 2	14353	Schizophrenia	495	289 (0.58)	181(0.37)	25 (0.05)	759 (0.77)	231 (0.23)	1.00	$\chi2 = 0.24$, $p = 0.62$	$p = 1.00$	$p = 0.96$
			Control	931	545 (0.59)	339 (0.36)	47(0.05)	1429(0.77)	433 (0.23)	(0.83–1.19)	$\chi2 = 0.38$, $p = 0.54$	$\chi2 = 0.0034$	$\chi2 = 0.0023$
rs12176601	9948019				TT	TA	AA	T	A				
	intron 2	11759	Schizophrenia	493	166(0.34)	244 (0.49)	83 (0.17)	576 (0.58)	410 (0.42)	1.21	$\chi2 = 0.17$, $p = 0.68$	$\mathbf{p = 0.029}$	$\mathbf{p = 0.021}$
			Control	917	375 (0.41)	403 (0.44)	139 (0.15)	1153 (0.63)	681 (0.37)	(1.03–1.41)	$\chi2 = 3.16$, $p = 0.08$	$\chi2 = 7.08$	$\chi2 = 5.35$
rs6966472	9933663				AA	AG	GG	A	G				
	intron 4	14356	Schizophrenia	493	381 (0.77)	103 (0.21)	9 (0.02)	865 (0.88)	121 (0.12)	0.73	$\chi2 = 0.43$, $p = 0.51$	$\mathbf{p = 0.023}$	$\mathbf{p = 0.0075}$
			Control	931	656 (0.70)	252 (0.27)	23 (0.02)	1564 (0.84)	298 (0.16)	(0.59–0.92)	$\chi2 = 0.04$, $p = 0.84$	$\chi2 = 7.59$	$\chi2 = 7.16$
rs17559978	9912136				CC	CT	TT	C	T				
	intron 7	21527	Schizophrenia	499	339 (0.68)	138 (0.28)	22 (0.04)	816 (0.82)	182 (0.18)	0.80	$\chi2 = 2.63$, $p = 0.10$	$\mathbf{p = 0.029}$	$\mathbf{p = 0.025}$
			Control	936	571 (0.61)	322 (0.34)	43 (0.05)	1464 (0.78)	408 (0.22)	(0.66–0.97)	$\chi2 = 0.08$, $p = 0.78$	$\chi2 = 7.11$	$\chi2 = 5.05$
rs17159577	9900238				CC	CT	TT	C	T				
	intron 10	11898	Schizophrenia	494	244 (0.49)	195 (0.39)	55 (0.11)	683 (0.69)	305 (0.31)	1.05	$\chi2 = 2.79$, $p = 0.09$	$p = 0.15$	$p = 0.60$
			Control	934	453 (0.49)	403 (0.43)	78 (0.08)	1309 (0.70)	559 (0.30)	(0.88–1.24)	$\chi2 = 0.77$, $p = 0.38$	$\chi2 = 3.78$	$\chi2 = 0.27$
rs17159556	9886562				GG	GT	TT	G	T				
	intron 10	13676	Schizophrenia	496	372 (0.75)	112 (0.23)	12 (0.02)	856(0.86)	136(0.14)	0.76	$\chi2 = 1.03$, $p = 0.31$	$\mathbf{p = 0.024}$	$\mathbf{p = 0.012}$
			Control	932	635 (0.68)	271 (0.29)	26 (0.03)	1541 (0.83)	323(0.17)	(0.61–0.94)	$\chi2 = 0.21$, $p = 0.65$	$\chi2 = 7.43$	$\chi2 = 6.29$
rs7800072	9863265				AA	AC	CC	A	C				
	exon 17	23297	Schizophrenia	502	342 (0.68)	140 (0.28)	20 (0.04)	824 (0.82)	180(0.18)	0.76	$\chi2 = 1.37$, $p = 0.24$	$\mathbf{p = 0.013}$	$\mathbf{p = 0.0069}$
	Lys701Gln		Control	934	563 (0.60)	327 (0.35)	44 (0.05)	1453(0.78)	415 (0.22)	(0.63–0.93)	$\chi2 = 0.16$, $p = 0.69$	$\chi2 = 8.67$	$\chi2 = 7.31$

[a] Chromosome position was established from the dbSNP database.
[b] Without Bonferroni's correction.
[c] HWE: Hardy–Weinberg equilibrium.
[d] GF: Genotype distribution frequency.
[e] AF: Allele distribution frequency.

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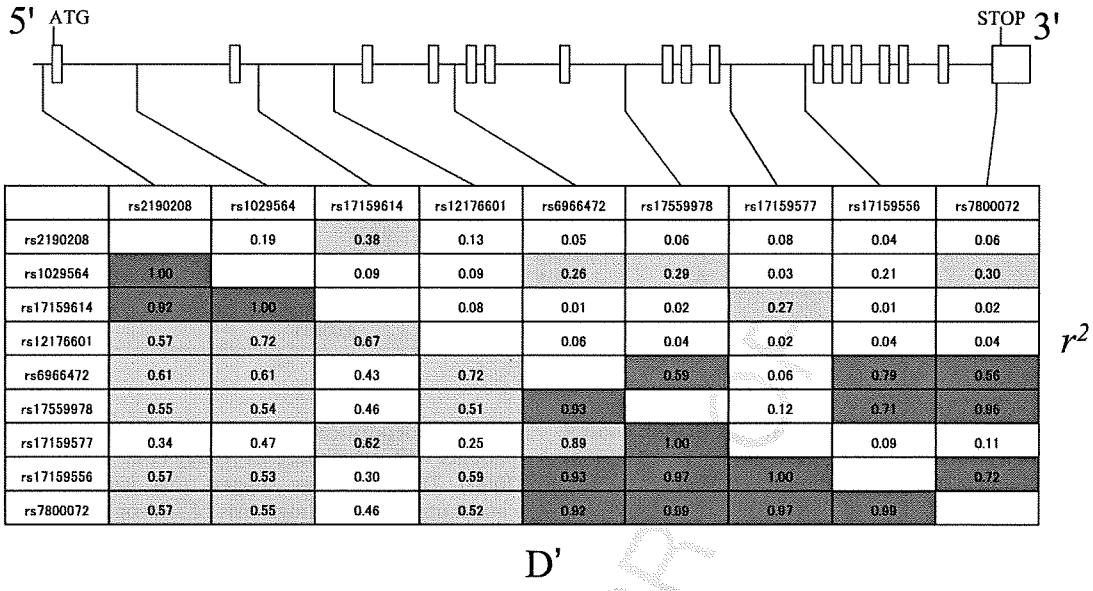

Fig. 1. The genetic structure of *SEMA3D* and location of the examined SNPs. The D′ and r^2 values between paired SNPs are shown in the diagram. The exonic regions are shown as white squares. The intensity of the box color corresponds to the strength of LD or r^2.

rs17159614, rs12176601, rs6966472, rs17559978, rs17159577, and rs17159556), several significant differences in genotype and allele distributions were observed (Table 1). To correct for multiple testing, we calculated the experiment-wide significance threshold required to keep the type I error rate at 5%. As a result, the corrected p value was calculated as 0.0085. The allelic associations with the SNPs rs7800072 (Lys701Gln) and rs6966472 remained significant after the correction (Table 1). Distinguishing between the carriers and the non-carriers with respect to the Gln701 allele for patients and controls, the protective effect became clearer ($p = 0.0033$).

The results of haplotype-based analyses are shown in Table 2. There were significant haplotypic associations of the SNPs in *SEMA3D* when comparing the schizophrenic patients and control subjects. In particular, the four-marker haplotype (rs2190208–rs1029564–rs17159614–rs121176601) showed a statistically significant association with schizophrenia (global permutation $p = 0.00001$). Concerning this haplotype analysis, global p values of 100,000 permutations, which corrected for multiple testing, were also significant. Furthermore, the haplotype frequency of GAGA was significantly higher in schizophrenia patients than in control subjects (0.376 and 0.291, permutation $p = 0.00005$), whereas those of GAGT, AAAA, and GCGA were significantly lower in schizophrenic patients than in controls (0.050 and 0.084, permutation $p = 0.0029$; 0.007 and 0.025, permutation $p = 0.0062$; 0.007 and 0.021, permutation $p = 0.020$, respectively) (Table 3).

When we performed stratified analysis of the data for rs7800072 by sex, a significant association was observed in women ($p = 0.0089$), but not in men ($p = 0.41$) (supplementary Tables 1 and 2). In the haplotype analysis, on the other hand, the four-marker haplotype (rs2190208–rs1029564–rs17159614–rs121176601) showed a statistically significant association in men (global permutation $p = 0.00001$), but was at a trend level in women (global permutation $p = 0.0699$). The haplotype frequency of GAGA

Table 2
Associations with schizophrenia of the 9 SNPs and haplotypes in SEMA3D.

SNP No.	dbSNP ID	Allele model	Haplotype p[a]							
		p value	2 Locus	3 Locus	4 Locus	5 Locus	6 Locus	7 Locus	8 Locus	9 Locus
SNP1	rs2190208	0.59								
			0.019							
SNP2	rs1029564	**0.011**		0.10						
			0.029		**0.00001**					
SNP3	rs17159614	0.96		**0.00002**		**0.00005**				
			0.0004		**0.0003**		**0.00007**			
SNP4	rs12176601	**0.021**		**0.0010**		**0.0001**		**0.0003**		
			0.035		**0.0006**		**0.0016**		**0.0007**	
SNP5	rs6966472	**0.0075**		0.053		**0.0004**		**0.0001**		**0.0007**
			0.023		0.098		**0.0001**		**0.0001**	
SNP6	rs17559978	**0.025**		**0.022**		0.061		**0.0004**		
			0.030		**0.025**		0.076			
SNP7	rs17159577	0.60		0.064		**0.024**				
			0.042		0.051					
SNP8	rs17159556	**0.012**		**0.028**						
			0.020							
SNP9	rs7800072	**0.0069**								

[a] global p value.

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Table 3
Estimated haplotype frequencies and association significance for SEMA3D.

Haplotype	rs2190208	rs1029564	rs17159614	rs12176601	% of individuals					
					Overall	Control	Schizophrenia	χ2	*p value*	Permutation *p* value
1	G	A	G	A	0.321	0.291	0.376	20.40	**0.0000063**	**0.000050**
2	A	A	A	T	0.207	0.201	0.219	1.21	0.27	0.28
3	G	C	G	T	0.190	0.199	0.172	2.98	0.085	0.089
4	A	A	G	T	0.142	0.143	0.139	0.10	0.75	0.76
5	G	A	G	T	0.072	0.084	0.050	11.23	**0.00080**	**0.0029**
6	A	A	G	A	0.034	0.036	0.031	0.37	0.54	0.59
7	A	A	A	A	0.019	0.025	0.007	10.75	**0.0010**	**0.0062**
8	G	C	G	A	0.016	0.021	0.007	7.55	**0.0060**	**0.020**

	χ2	*p value*	Permutation *p* value	Replications
Global	46.07	0.000000085	**0.00001**	100000

was significantly higher in schizophrenia patients than in control subjects in both men (0.368 and 0.272, permutation $p = 0.00053$) and women (0.384 and 0.302, permutation $p = 0.003$).

4. Discussion

Our results provide the first evidence for the possible involvement of *SEMA3D* in the pathogenesis of schizophrenia. With respect to the non-synonymous (Lys701Gln) polymorphism, we found a significant preponderance of the Lys/Lys genotype and the Lys701 allele in schizophrenia patients compared with control subjects. In the haplotype-based analyses, we also obtained evidence for an association between *SEMA3D* and schizophrenia. Interestingly, the most significant haplotype, rs2190208–rs1029564–rs17159614–rs121176601, does not include rs7800072 (Lys701Gln) (see Fig. 1). Therefore, it is likely that at least one functional polymorphism other than rs7800072, which is in linkage disequilibrium to the haplotype, could be responsible for susceptibility to schizophrenia. In stratified analysis for rs7800072 by sex, the frequency of the Gln701 allele was significantly lower in schizophrenia patients than in control subjects in women (0.17 and 0.23, $p = 0.0088$) (supplementary Table 2). Likewise, this was also lower in men, but was not statistically significant (0.18 and 0.20, $p = 0.41$) (supplementary Table 1). Regarding analysis of the four-marker haplotype (rs2190208–rs1029564–rs17159614–rs121176601), there remained a statistical significance in men (global permutation $p = 0.00001$) and a tendency in women (global permutation $p = 0.0699$). In addition, the frequency of the most major haplotype (GAGA) was significantly higher in schizophrenia patients than in control subjects in both sexes. These inconsistent results between males and females are likely to have arisen from the lack of statistical power after dividing the sexes.

The neurodevelopmental hypothesis of schizophrenia proposes that abnormalities of brain development are involved in the pathogenesis of schizophrenia (Conrad and Scheibel, 1987; Weinberger, 1987; Murray, 1994; Waddington et al., 1998). In early brain developmental stages, a number of semaphorins play important roles in axonal repulsion, axonal attraction, neuronal cell migration, and axon pruning (reviewed in Kruger et al., 2005; Waimey and Cheng, 2006; Halloran and Wolman, 2006; Mann et al., 2007). Indeed, SEMA3D has been shown to act in axon guidance and cell migration during neuronal development (Wolman et al., 2004, 2007; Liu et al., 2004; Liu and Halloran, 2005; Sakai and Halloran, 2006; Takahashi et al., 2009). With respect to neuronal cell migration, neuronal disarray and abnormal migration in the neocortical white matter were reported in postmortem studies of patients with schizophrenia (Jakob and Beckmann, 1986; Akbarian et al., 1993). Regarding pruning, Feinberg proposed that schizophrenia may arise from excessive synaptic pruning during adolescence (Feinberg, 1982; Keshavan et al., 1994). Indeed, decreased density of dendritic spines was observed in the prefrontal cortex of patients with schizophrenia (Garey et al., 1998; Glantz and Lewis, 2000). These findings suggest that variants of *SEMA3D* may contribute to the pathogenesis of schizophrenia through affecting development of neural networks. The genotypic difference based on the Lys701Gln polymorphism of *SEMA3D* might lead to developmental differences in the brain; the Gln701 carriers would exhibit intrinsically greater protective effects against the development of schizophrenia than the Gln701 non-carriers. Although *SEMA3D* has not yet been well-studied, *SEMA3A* has been investigated in detail. In particular, an increase in the expression of SEMA3A has previously been associated with schizophrenia (Eastwood et al., 2003). Moreover, *PLXNA2*, which encodes one of the receptors for class 3 semaphorins, was identified as a candidate gene for schizophrenia in a genome-wide association study (Mah et al., 2006). Currently, this association is also supported by the meta-analysis of Allen et al. (2008). SEMA3A and SEMA3D belong to the same class and share the most similarity with each other of the class 3 semaphorin genes (Luo et al., 1995). These findings further strengthen the evidence for a possible role of *SEMA3D* in the development of schizophrenia.

It is possible that the amino acid change (Lys701Gln) may affect the function of SEMA3D protein and that this results in susceptibility to schizophrenia. Indeed, this is a substitution from a large and basic amino acid (Lys) to a medium-sized and polar one (Gln). This is likely to lead to functional differences between the two types of SEMA3D. One possibility is that this substitution might result in conformational change of SEMA3D and influence its affinity for its receptors. Another possibility is that the Lys701 and Gln701 variants of SEMA3D have different cellular localization. The basic domain of class 3 semaphorins electrostatically interacts with the proteoglycan components of the extracellular matrix (De Wit et al., 2005) and the granule matrix (de Wit et al., 2009). The substitution from the basic Lys701 to the non-basic Gln701 may affect such interactions between SEMA3D and these matrices. Alteration of the extracellular matrix may modify distribution of SEMA3D in neurons, and that of the granule matrix may affect secretion from secretory vesicles. The class 3 semaphorins not only act as axon guidance cues but also have key roles in synaptic formation and function. Therefore, these modified interactions could impact on the establishment of synaptic contacts and the formation of new synapses. Although the amino acid substitution (Lys701Gln) was predicted to be benign by Polyphen (http://genetics.bwh.harvard.edu/pph/) and SIFT (http://sift.jcvi.org/) programs, its actual effects should be elucidated by cell biological or biochemical approaches.

Accumulating evidence suggests that the semaphorins are regulatory factors of tumor progression and modulators of angiogenesis (reviewed in (Neufeld and Kessler, 2008) and (Capparuccia and Tamagnone, 2009)). Recently, SEMA3D was also reported to

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possess anti-tumorigenic and anti-angiogenic properties (Kigel et al., 2008). The hypoactivity of *SEMA3D* could be linked to increased incidence of cancer. Previous studies and reviews have partially supported the idea that the incidence of cancer in patients with schizophrenia is reduced compared with the general population (Grinshpoon et al., 2005; Dalton et al., 2005; Catts et al., 2008). It is possible that semaphorins are related to the development of schizophrenia and also contribute to the associated lower incidence of cancer, and this topic warrants further investigation.

In conclusion, we found a significant association between the Lys701Gln polymorphism of *SEMA3D* and schizophrenia. In addition, the haplotype rs2190208–rs1029564–rs17159614–rs121176601, not including the Lys701Gln variant, was shown to be associated with schizophrenia, which suggests that some other polymorphisms of *SEMA3D* play a role in the pathogenesis of schizophrenia. Taking the previous molecular and developmental findings together with the present genetic findings, *SEMA3D* appears to be a promising candidate gene related to susceptibility to schizophrenia.

Conflict of interest

All authors declare no conflict of interest that could influence their work.

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Contributors

T.F. designed the study, performed genotyping of *SEMA3D*, made statistical analysis, managed literature search, interpreted the data, and wrote the manuscript. H.U. and N.Y. took part in genotyping. H. H., M.T., M.I., K.A., and T.H. collected samples and gave comments to the manuscript. H.K. organized recruitment and genotyping of schizophrenic patients and control subjects, and took part in analyzing the data and writing the manuscript.

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Appendix. Supplementary data

Supplementary data associated with this article can be found in the online version at doi: 10.1016/j.jpsychires.2010.05.004.

References

Akbarian S, Bunney Jr WE, Potkin SG, Wigal SB, Hagman JO, Sandman CA, Jones EG. Altered distribution of nicotinamide-adenine dinucleotide phosphate-diaphorase cells in frontal lobe of schizophrenics implies disturbances of cortical development. Archives of General Psychiatry 1993;50:169–77.

Allen NC, Bagade S, McQueen MB, Ioannidis JP, Kavvoura FK, Khoury MJ, et al. Systematic meta-analyses and field synopsis of genetic association studies in schizophrenia: the SzGene database. Nature Genetics 2008;40:827–34.

American Psychiatric Association. Diagnostic and statistical manual of mental disorders. 4th ed. Washington, DC: American Psychiatric Association (APA); 1994.

Budel S, Shim SO, Feng Z, Zhao H, Hisama F, Strittmatter SM. No association between schizophrenia and polymorphisms of the PlexinA2 gene in Chinese Han Trios. Schizophrenia Research 2008;99:365–6.

Capparuccia L, Tamagnone L. Semaphorin signaling in cancer cells and in cells of the tumor microenvironment–two sides of a coin. Journal of Cell Science 2009;122:1723–36.

Catts VS, Catts SV, O'Toole BI, Frost AD. Cancer incidence in patients with schizophrenia and their first-degree relatives–a meta-analysis. Acta Psychiatrica Scandinavica 2008;117:323–36.

Clark HF, Gurney AL, Abaya E, Baker K, Baldwin D, Brush J, et al. The secreted protein discovery initiative (SPDI), a large-scale effort to identify novel human secreted and transmembrane proteins: a bioinformatics assessment. Genome Research 2003;13:2265–70.

Conrad AJ, Scheibel AB. Schizophrenia and the hippocampus: the embryological hypothesis extended. Schizophrenia Bulletin 1987;13:577–87.

Dalton SO, Mellemkjaer L, Thomassen L, Mortensen PB, Johansen C. Risk for cancer in a cohort of patients hospitalized for schizophrenia in Denmark, 1969–1993. Schizophrenia Research 2005;75:315–24.

De Wit J, De Winter F, Klooster J, Verhaagen J. Semaphorin 3A displays a punctate distribution on the surface of neuronal cells and interacts with proteoglycans in the extracellular matrix. Molecular and Cellular Neurosciences 2005;29:40–55.

de Wit J, Toonen RF, Verhage M. Matrix-dependent local retention of secretory vesicle cargo in cortical neurons. The Journal of Neuroscience 2009;29:23–37.

Eastwood SL, Law AJ, Everall IP, Harrison PJ. The axonal chemorepellant semaphorin 3A is increased in the cerebellum in schizophrenia and may contribute to its synaptic pathology. Molecular Psychiatry 2003;8:148–55.

Ekelund J, Lichtermann D, Hovatta I, Ellonen P, Suvisaari J, Terwilliger JD, et al. Genome-wide scan for schizophrenia in the Finnish population: evidence for a locus on chromosome 7q22. Human Molecular Genetics 2000;9:1049–57.

Feinberg I. Schizophrenia: caused by a fault in programmed synaptic elimination during adolescence? Journal of Psychiatric Research 1982;17:319–34.

Fujii T, Iijima Y, Kondo H, Shizuno T, Hori H, Nakabayashi T, et al. Failure to confirm an association between the PLXNA2 gene and schizophrenia in a Japanese population. Progress in Neuro-Psychopharmacology & Biological Psychiatry 2007;31:873–7.

Fujii T, Nakao F, Shibata Y, Shioi G, Kodama E, Fujisawa H, Takagi S. Caenorhabditis elegans PlexinA, PLX-1, interacts with transmembrane semaphorins and regulates epidermal morphogenesis. Development 2002;129:2053–63.

Fujisawa H. Discovery of semaphorin receptors, neuropilin and plexin, and their functions in neural development. Journal of Neurobiology 2004;59:24–33.

Garey LJ, Ong WY, Patel TS, Kanani M, Davis A, Mortimer AM, et al. Reduced dendritic spine density on cerebral cortical pyramidal neurons in schizophrenia. Journal of Neurology, Neurosurgery, and Psychiatry 1998;65:446–53.

Glantz LA, Lewis DA. Decreased dendritic spine density on prefrontal cortical pyramidal neurons in schizophrenia. Archives of General Psychiatry 2000;57:65–73.

Grinshpoon A, Barchana M, Ponizovsky A, Lipshitz I, Nahon D, Tal O, et al. Cancer in schizophrenia: is the risk higher or lower? Schizophrenia Research 2005;73:333–41.

Halloran MC, Wolman MA. Repulsion or adhesion: receptors make the call. Current Opinion in Cell Biology 2006;18:533–40.

Idol JR, Addington AM, Long RT, Rapoport JL, Green ED. Sequencing and analyzing the t(1;7) reciprocal translocation breakpoints associated with a case of childhood-onset schizophrenia/autistic disorder. Journal of Autism and Developmental Disorders 2008;38:668–77.

Jakob H, Beckmann H. Prenatal developmental disturbances in the limbic allocortex in schizophrenics. Journal of Neural Transmission 1986;65:303–26.

Keshavan MS, Anderson S, Pettegrew JW. Is schizophrenia due to excessive synaptic pruning in the prefrontal cortex? the Feinberg hypothesis revisited. Journal of Psychiatric Research 1994;28:239–65.

Kigel B, Varshavsky A, Kessler O, Neufeld G. Successful inhibition of tumor development by specific class-3 semaphorins is associated with expression of appropriate semaphorin receptors by tumor cells. PLoS One 2008;3:e3287.

Kruger RP, Aurandt J, Guan KL. Semaphorins command cells to move. Nature Reviews. Molecular Cell Biology 2005;6:789–800.

Li J, Ji L. Adjusting multiple testing in multilocus analyses using the eigenvalues of a correlation matrix. Heredity 2005;95:221–7.

Liu Y, Berndt J, Su F, Tawarayama H, Shoji W, Kuwada JY, Halloran MC. Semaphorin3D guides retinal axons along the dorsoventral axis of the tectum. The Journal of Neuroscience 2004;24:310–8.

Liu Y, Halloran MC. Central and peripheral axon branches from one neuron are guided differentially by Semaphorin3D and transient axonal glycoprotein-1. The Journal of Neuroscience 2005;25:10556–63.

Luo Y, Raible D, Raper JA. Collapsin: a protein in brain that induces the collapse and paralysis of neuronal growth cones. Cell 1993;75:217–27.

Luo Y, Shepherd I, Li J, Renzi MJ, Chang S, Raper JA. A family of molecules related to collapsin in the embryonic chick nervous system. Neuron 1995;14:1131–40.

Mah S, Nelson MR, Delisi LE, Reneland RH, Markward N, James MR, et al. Identification of the semaphorin receptor PLXNA2 as a candidate for susceptibility to schizophrenia. Molecular Psychiatry 2006;11:471–8.

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