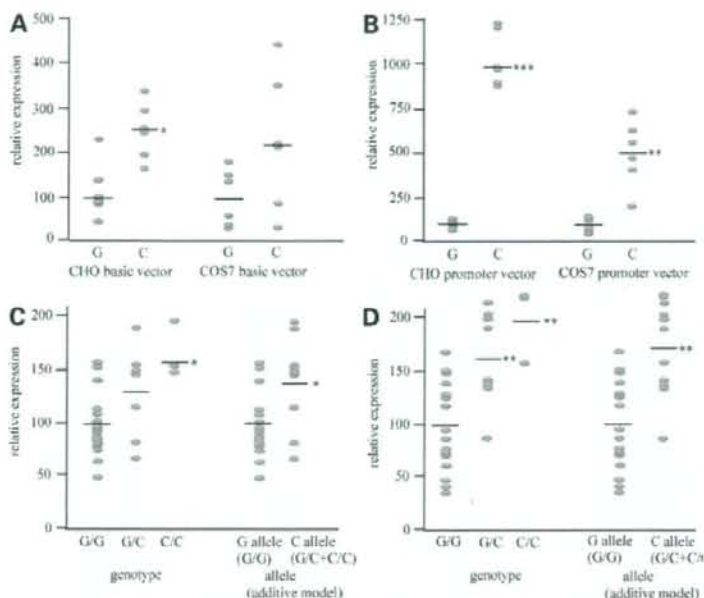

Figure 2. *In vitro* and *in vivo* expression assays. (**A**) Promoterless vector (basic vector) and (**B**) promoter vector in CHO and COS7 cells. Firefly luciferase activities were normalized with Renilla luciferase activities. Relative expression was calculated as 100 for the major allele (G allele) of *rs28365839*. $^*P <$ 0.05, $^{**}P < 0.01$, $^{***}P < 0.001$ (in Student's *t*-test). Results of (**C**) real-time RT–PCR and (**D**) western blot analysis. Expression levels of 14-3-3epsilon RNA and protein were normalized with GAPDH expression. Relative expression was calculated as 100 for the major allele homozygous genotype (G/G genotype) or major allele (G allele) of *rs28365839*. Number of individuals with the distinct genotypes of *rs28365839* were 16 for G/G, 8 for G/C and 3 for C/C. $^*P <$ 0.05, $^{**}P < 0.01$ (in *post hoc* comparison with the Dunnett test for genotype-wise analysis, and *t*-test for allele-wise analysis).

Moderately enhanced anxiety-like behavior in *Ywhae*$^{+/-}$ mice in the elevated plus-maze test

To examine the effect of 14-3-3epsilon deficit on anxiety-like behavior, *Ywhae*$^{+/-}$ mice and their wild-type littermates were analyzed in light/dark transition and elevated plus-maze tests. In light/dark transition, no significant difference was observed between *Ywhae*$^{+/-}$ mice and their wild-type littermates (Table 3). In the elevated plus-maze test, *Ywhae*$^{+/-}$ mice showed a smaller number of total entries ($P = 0.0075$) (Fig. 3E), increased time spent on closed arms ($P = 0.0195$) (Fig. 3F) and decreased time spent on center area ($P =$ 0.0012) (Table 3). A significant difference was not observed in the number of entries onto open arms, total distance travelled or time spent on open arms ($P = 0.2044$, 0.1071, 0.3798, respectively) (Table 3). Thus, it is conceivable that *Ywhae*$^{+/-}$ mice have moderately enhanced anxiety-like behavior that could be detected only by the elevated plus-maze test but not by the light/dark transition or by the open-field tests.

DISCUSSION

Association between *YWHAE* and schizophrenia

In this study, we have identified *YWHAE*, the gene encoding 14-3-3epsilon, which forms a complex with DISC1 *in vivo*, as a possible susceptibility gene for schizophrenia. Genetic and expression evidence indicates that the SNP in 5′ flanking region (*rs28365859*) is associated with schizophrenia through influencing the expression level of *YWHAE*. Subjects with the C allele of *rs28365859* were thought to have a reduced risk of schizophrenia [odds ratio of combined subjects = 0.76 (95% confidence interval: 0.68-0.86)]. Our sample size was relatively large (3157samples consisting of 706 first-set and 2451 second-set samples: 1429 schizophrenics and 1728 controls), making our results reliable. In addition, another research assessing the genetic association of *YWHAE* with suicide victims [two of SNPs (*rs3752826* and *rs9393*) are identical SNPs in our study and another SNP (*rs1532976*) can be captured by *rs3752826* using HapMap information] can support our results, since it showed the same trends in the distributions of MAFs (MAFs of these SNPs were higher in controls than in cases) (34). However, a couple of limitations should be outlined. First, our results that show statistical significances may be derived from unknown population stratification, since Genomic Control was not included in this analysis. Secondly, there could be a possible effect of differential age distribution between cases and controls in the association analysis.

The *in vitro* luciferase assay suggests that the C allele might act as an enhancer, since significant luciferase induction could not be seen with the use of a promoterless vector, but luciferase activity (LA) could be assayed from the vector containing a promoter. Further, *in vivo* expression assays of RNA and

Table 3. Comprehensive behavioral test battery

Test		*Ywhae*$^{+/+}$	*Ywhae*$^{+/-}$	*P*-value	*F* value
General health					
Weight (g)		28.6	29.682	0.0262*	1.335
Rectal temperature (°C)		37.033	36.688	0.0435*	4.406
Pain test					
Hot plate (latency, s)		6.206	5.053	0.1142	2.633
Motor tests					
Grip strength (*n*)		1.044	1.085	0.2825	1.194
Wire hang (latency to fall, s)		60	50	0.0234*	5.65
Rotarod (latency to fall, s; average of six trials)		161.759	182.618	0.3391	0.941
Anxiety-like behavior					
Light/dark transition					
Distance travelled (cm)	Light side	484.983	617.782	0.0728	3.434
	Dark side	1095.389	1099.288	0.97	0.001
Stay time in light side (s)		214.972	231.176	0.6043	0.274
Transitions (times)		35.111	33.588	0.6827	0.17
Latency to light side (s)		31.444	34.941	0.6683	0.187
Elevated plus-maze					
Number of entries (times)		32.556	25.118	0.0075**	8.126
Entries onto open arms (%)		31.824	26.648	0.2044	1.677
Distance travelled (cm)		1323.722	1194.329	0.1071	2.744
Time on open arms (%)		15.269	12.971	0.3798	0.793
Time on closed arms (%)		50.87	63.196	0.0195*	6.034
Time on center area (%)		35.034	23.283	0.0012*	12.495
Depression model					
Porsolt forced swim (immobility, %)	Day 1	59.369	65.648	0.0661	3.614
	Day 2	77.026	78.564	0.6256	0.243
Tail suspension (immobility, %)		26.194	22.774	0.6267	0.241
Locomotor activity					
Open field					
Total distance travelled (cm)		8745.222	9258.941	0.5822	0.309
Vertical activity (times)		208.722	393.824	0.047*	4.259
Center time (s/min)		1.432	1.107	0.6505	0.209
Stereotypic counts (times)		7260.944	6124.118	0.2251	1.528
Sensory motor gating					
Acoustic startle response		3.021	2.704	0.32	1.02
Prepulse inhibition (startle stimulus, %)					
110-dB startle		48.887	47.697	0.8496	0.037
120-dB startle		13.566	16.6	0.5617	0.344
Working memory					
8-arm radial maze					
Training					
Different arm choice in first 8 entries (times)		6.209	6.016	0.3325	0.967
Revisiting errors (times)		6.12	7.613	0.1557	2.11
Delay 30 s					
Different arm choice in first 8 entries (times)		6.5	6.471	0.8972	0.017
Revisiting errors (times)		3.417	3.676	0.7599	0.095
Delay 120 s					
Different arm choice in first 8 entries (times)		6	5.882	0.6476	0.213
Revisiting errors (times)		4.944	6.735	0.1715	1.954
Delay 300 s					
Different arm choice in first 8 entries (times)		6.167	5.971	0.5077	0.448
Revisiting errors (times)		3.778	6.294	0.0229*	5.698
Reference memory					
T-maze (correct, %)	Training	80.648	77.157	0.0696	3.519
	Reverse	61.759	59.314	0.4567	0.567
Cued and contextual fear conditioning					
Conditioning (freezing, %)		28.324	29.29	0.7581	0.096
Context testing (freezing, %)		50.998	46.611	0.5754	0.32
Cued testing with altered context freezing, %)		53.641	52.926	0.8342	0.045
Social interaction					
Total duration of contact (s)		118.386	153.383	0.1239	2.776
Number of contacts (times)		49.429	53.333	0.4968	0.494
Total duration of active contacts (s)		14.257	18.733	0.0809	3.693
Mean duration/contact		2.443	3.017	0.2889	1.241
Distance travelled (cm)		2789.357	2882.167	0.7206	0.135

Behavioral test battery was performed in the following order: general health/neurological screen, wire hang, grip strength test, light/dark transition, open field, elevated plus-maze, hot plate, social interaction (novel environment), rotarod, prepulse inhibition, Porsolt forced swim, eight arm radial maze, T-maze, cued and contextual fear condition test, latent inhibition, tail suspension test.
*$P < 0.05$, **$P < 0.01$.

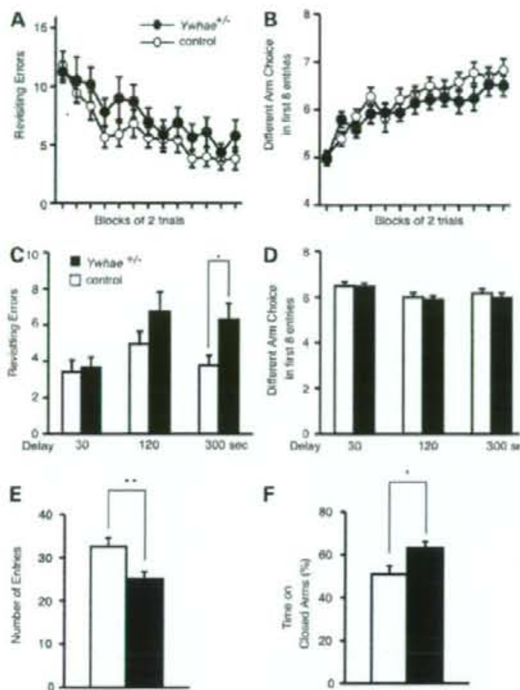

Figure 3. Behavioral abnormality of *Ywhae*$^{+/-}$ mice. (**A–D**) Working memory test of *Ywhae*$^{+/-}$ mice in the 8-arm radial maze (A and B). Total number of revisiting errors (A) and the number of different arms chosen in the first 8-arm visits (B) across training were counted. Data are presented as 2-day/trial averages. (C and D) Total number of revisiting errors (C) and the number of different arms chosen in the first 8-arm visits (D) of mice after training; exposure to delays of 30, 120 or 300 s after four pellets had been taken were counted (see *Materials and Methods*). (**E** and **F**) Anxiety-like behavior test of *Ywhae*$^{+/-}$ mice in the elevated plus-maze. (E) Number of total entries. (F) Time spent on closed arms. Number of total entries was lower and time spent on closed arms were greater in *Ywhae*$^{+/-}$ mice than in controls. Asterisks indicate a difference from the values of control mice. $^{*}P < 0.05$, $^{**}P < 0.01$.

protein in peripheral blood samples clarified the functional relevance of this SNP: Subjects who were heterozygous and homozygous with the C allele had higher expression of 14-3-3epsilon. Of note, our samples were control subjects not on medication; therefore, we could avoid the bias related to drug effects, which may be seen when studying schizophrenia subjects.

Also, haplotype trend regression analysis showed that the haplotypes consisted of five SNPs located in 5′ flanking region (*rs28365859*) and intron1 (*rs11655548*, *rs2131431*, *rs1873827* and *rs12452627*) were correlated with the expression level of *YWHAE*, whereas each SNP in intron 1 was not correlated with the expression. Therefore, we speculate that this significant result in haplotype-wise analysis may be derived mainly from the effects of *rs28365859*.

We analyzed for the homology of genome sequence between human and mice *Ywhae* gene using 500 bp upstream region from start ATG. About 200 bp upstream region from start ATG shows high identity, however, a region containing *rs2836589* SNP does not show homology. This result suggests that this SNP is not evolutionally conserved. We searched for functional motif on the sequence in the 5′ upstream region of *YWHAE* including *rs28365859* using TESS: Transcription Element Search System (http://www.cbil.upenn.edu/cgi-bin/tess/tess). In minor allele (C allele), ubiquitously expressed cellular upstream stimulatory factor (USF) -interacting motif 'CCA**C**GT' was detected in this in silico analysis. This result may explain a possible functional effect of this SNP, an upregulation of 14-3-3epsilon in C allele-harboring people, however, further analysis would be needed to provide definitive conclusion.

Role of 14-3-3epsilon in neuronal development

Several observations of the postmortem brain suggest that alterations in neuronal cell migration, and synaptic, dendritic and axonal organizations occur in schizophrenia patients (35,36). *Ywhae*$^{+/-}$ mice show milder migration defects in both the cortex and the hippocampus, whereas *Ywhae*$^{-/-}$ mice display severe neuronal migration defects (29). Primary hippocampal neurons from *Ywhae*$^{-/-}$ mice display shorter axons and a defect in accumulation of the NUDEL/LIS1 complex in the distal part of axons (29). We confirmed that knockdown of 14-3-3epsilon by RNAi impairs not only the NUDEL/LIS1 complex transport into axons but also axon elongation (data not shown). Previously, we identified 14-3-3epsilon as an interacting molecule of *DISC1* (22). DISC1 is required to transport the NUDEL/LIS1/14-3-3epsilon complex into axons (22). Of note, depletion of endogenous DISC1 by RNAi results in a severe neuronal migration defect in the developing neocortex via regulation of the dynein complex (37). These results and reports suggest that both DISC1 and 14-3-3epsilon are required for neuronal development via transport of the NUDEL/LIS1 complex. To clarify the functional relationship between 14-3-3 epsilon and DISC1 on neuronal development via transport of the NUDEL/LIS1 complex, further genetic analysis using knockout mice will be required.

Cognitive dysfunction of *Ywhae*$^{+/-}$ mice

Ywhae$^{+/-}$ mice, in which the expression of 14-3-3epsilon protein was reduced to ~50% compared with their wild-type littermates, showed weak cognitive dysfunction specifically in working memory (Table 3). Interestingly, missense mutant mice of the *DISC1* gene show defects in working memory (38). Reduction of DISC1 or 14-3-3epsilon results in developmental defects of hippocampal neurons. These results and reports suggest that impairment of DISC1 or 14-3-3epsilon cause neuronal developmental defects, that result in cognitive dysfunction. Interestingly, impairment of working memory is one of the prominent features of schizophrenia symptomatology (39–41). Non-synonymous polymorphism of *DISC1* that consists of a serine to cysteine substitution at codon 704 (*DISC1*Ser704Cys) is reported to correlate with variations in hippocampal size and cognitive function including working memory, and is associated with

schizophrenia (42). Although relation between 14-3-3epsilon and cognitive function in human is not known, 14-3-3epsilon could be implicated in cognitive function that is associated with DISC1. Another prominent feature of schizophrenia symptomatology, prepulse inhibition (43), did not differ in *Ywhae*$^{+/-}$ mice compared with their wild-type littermates (Table 3). Schizophrenia is a complex disorder with a variety of pathology and risk factor genes. It is a reasonable assumption that modification of a single gene does not mimic all features of schizophrenia symptomatology. We think that our results using *Ywhae*$^{+/-}$ mice partly support our genetic data. However, further analysis would be required to clarify a role of 14-3-3epsilon on cognitive functions and functional relationship between *YWHAE* and *DISC1*.

YWHAE as a possible susceptibility gene for schizophrenia

In this study, we found that a SNP of *YWHAE* that correlates the expression of 14-3-3epsilon is associated with schizophrenia, and that this SNP would reduce the risk of schizophrenia. Perhaps, increased 14-3-3epsilon expression in humans affected by the identified SNP is protective, whereas decreased 14-3-3epsilon expression due to 50% reduction by heterozygous knockout in mice results in behavioral deficits. At this point, we do not know why higher expression levels of 14-3-3epsilon reduce the risk of schizophrenia, or why lower expression levels of this gene result in increase of the risk in human and behavioral changes in mice. By its susceptibility genes, schizophrenia seems to be a complex disorder with multiple symptoms and genetic risk factors. We predict that schizophrenia would be divided into several classes by its susceptibility genes. Each class would have its own molecular/signaling pathway that plays important roles in the pathogenesis. DISC1 and its interacting molecules are required in neuronal developments and adult neurongenesis (44), and would play critical roles in pathogenesis of specific classes of schizophrenia. In other classes of schizophrenia, the DISC1-pathway would not be implicated in the pathogenesis. Some genes could have redundant functions. 14-3-3epsilon is a member of adaptor proteins that interact with phosphorylated serine or threonine residue of target proteins. More than 100 of 14-3-3-binding partners involved in signal transduction, cell cycle regulation, apoptosis, stress responses and malignant transformation have been identified (45). Proteomic analysis of synapse revealed that 14-3-3epsilon forms a complex with NMDA receptor (46). Placing these results and reports in the context of the pathogenesis of schizophrenia, 14-3-3epsilon could be a susceptibility gene of not only DISC1-implicated, but also wide range of schizophrenia because of its wide variety of interacting partners. 14-3-3epsilon would be a key molecule to understand molecular mechanisms of susceptibility genes for schizophrenia.

MATERIALS AND METHODS

Subjects in genetic association analyses

In the genetic association analyses, two independent sets of subjects were examined. The first screening analysis included 364 patients with schizophrenia (188 male and 176 female; mean age ± SD 42.5 ± 14.8 years) and 342 healthy controls (191 male and 151 female; 35.0 ± 13.6 years). Patients for the second confirmation analysis included 1065 patients with schizophrenia (562 male and 503 female; 48.9 ± 14.7 years) and 1386 controls (714 male and 672 female; 42.6 ± 14.6 years). All subjects were unrelated to each other and reported to be of Japanese ethnicity. Forty patients with schizophrenia were used as subjects for a mutation search; these subjects were also included in the first-set screening scan. The schizophrenia patients were diagnosed according to criteria in the *Diagnostic and Statistical Manual of Mental Disorders, Fourth Edition* after at least two experienced psychiatrists reached consensus on the diagnosis on the basis of unstructured interviews and review of medical records. All healthy controls were also psychiatrically screened on the basis of unstructured interviews; to exclude subjects with any brain disorder, or psychotic disorder, or who had first-degree relatives with psychotic disorders, trained psychiatrist interviewed them to assess current and/or past mental states (psychotic, mood, anxiety, obsessive-compulsive symptoms) and family history. After description of the study, written informed consent was obtained from each subject. This study was approved by the ethics committees at Fujita Health University, Teikyo University, Okayama University, and Nagoya University Graduate School of Medicine.

SNP selection and genotyping

For LD-based association analysis using the first set of screening samples, we first consulted the HapMap and dbSNP databases to pick-up 'tagging SNPs'. From the HapMap database (Data Release #21: population JPT: MAF of >0.05: regions 8275000.8320000 for NUDEL, 2440000.2537000 for *LIS1*, 1193000.1256000 for *YWHAE*, 70823000.70917000 for *GRB2*, 56227000.56266000 for *KIF5A*), we selected a total of 27 tagging SNPs (one SNP for *NUDEL*, six SNPs for *LIS1*, nine SNPs for *YWHAE*, six SNPs for *GRB2* and five SNPs for *KIF5A*) with a threshold criterion of $r^2 > 0.8$ in pairwise tagging mode using Tagger software (47). Two SNPs (one for *NUDEL*, rs3744652 and one for *YWHAE*, rs34041110) were added for denser mapping.

All SNPs were genotyped by TaqMan assays, primer extension using dHPLC and polymerase chain reaction–restriction fragment length polymorphism assays as described previously (48). More detailed assay information can be found in Supplementary Material, Table S1.

Mutation search

After we detected significant association of *YWHAE* in screening samples, we used dHPLC analysis for a mutation search, the details of which are described in a previous paper (48). Primer pairs (Supplementary Material, Table S2) were designed with the use of information from the GenBank sequence (accession number: NT 010718.15) into 10 amplified regions, which covered all the coding regions, the branch sites and the 5′ flanking region 1026 bp upstream from the initial exon of *YWHAE*.

In vivo and *in vitro* expression assays

We used a dual-luciferase assay, real-time RT–PCR and western blot analysis to examine the influence of SNP *rs28365859* in the 5′ flanking region on expression levels of *YWHAE*. For the dual-luciferase assay, 497-bp fragments that included *rs28365859* were PCR amplified (Supplementary Material, Table S1). Genomic DNAs with identified genotypes were used as templates, and PCR products of either genotypes were cloned into a pGL3-basic vector and a pGL3-promoter vector (Promega, WI). These vectors with both alleles, the Renilla luciferase vector and the phRL-TK vector, were transiently transfected into Chinese hamster ovary (CHO) cells and COS-7 cells with the use of Lipofectamine 2000™ (Invitrogen, CA). All inserts were sequenced to confirm the containing alleles. After 48 h, cell extracts were prepared and assayed for firefly LA (LA_F) and Renilla LA (LA_R) as described by the manufacturer (Pikka-Gene Dual SeaPansy™ Luminescence Kit, Tokyo Ink, Japan) on a Fluoroskan Ascent FL (Thermo Labsystems, Finland).

For *in vitro* assays (real-time RT–PCR and western blot analysis), we processed and analyzed a total of 27 peripheral blood samples from normal control subjects to determine the amount of *YWHAE* transcript or protein: 16 subjects with homozygous major alleles (G/G genotype: 7 male and 9 female; 32.6 ± 6.4 years) in *rs28365859*; 8 subjects with heterozygous major alleles (G/C genotype: 4 male and 4 female; 33.5 ± 7.7 years) and 3 subjects with homozygous minor alleles (C/C genotype: 1 male and 2 female; 51.3 ± 17.0 years). These subjects were healthy controls who had not received any medication within at least 1 month before the collection of RNA and protein.

In the real-time RT–PCR assay, total RNA was isolated with the use of a QIAamp RNA Blood Mini Kit (QIAGEN, Inc., CA). Complementary DNA was generated with the use of a High-Capacity cDNA Archive Kit (Applied Biosystems). Real-time PCR constituents were 50 ng DNA, 2× TaqMan Universal Master Mix and 20× primer/probe mix (Hs00356749_g1, Applied Biosystems) in a 50-μl final volume. The amplification was done according to the manufacturer's instructions, and signals were recorded during PCR with the use of an ABI PRISM 7900 instrument. All gene expression results were normalized to the expression of glyceryl aldehyde-3-phosphate dehydrogenase (*GAPDH*).

In the western blot analysis, lymphocytes were purified (Axis-Shield, Oslo, Norway) and protein concentrations were determined with bovine serum albumin as the reference protein. The antibody against 14-3-3epsilon and alpha-tubulin were purchased (Santa Cruz Biotechnology, Inc., Santa Cruz, CA). Proteins were subjected to SDS–PAGE, followed by immunoblot analyses with anti-14-3-3epsilon or anti-alpha-tubulin antibody. The amount of 14-3-3epsilon was detected by chemiluminescence in a linear range using serial dilutions of standards and was estimated with Densitograph (ATTO, Tokyo, Japan). Alpha-tubulin was used as the standard for quantification. The results of these *in vivo* and *in vitro* expression assays were representative of three independent experiments.

Animals and experimental design

Ywhae$^{+/-}$ mice and their wild-type littermates were obtained as previously reported (29). Genetic background of mice is mixed 129/S6 × NIH Black Swiss. All behavioral tests (8-arm radial maze test, elevated plus-maze test, T-maze test, light/dark transition test and startle response/prepulse inhibition tests) were carried out with male mice that were 9–10 weeks old at the start of the testing. Heterozygous knockout mice and wild-type littermates were compared in experiments. Mice were housed in a room with a 12-h light/dark cycle (lights on at 7:00 a.m.) with access to food and water ad libitum. Behavioral testing was performed between 9:00 a.m. and 6:00 p.m. After the tests, all apparatus was cleaned with super hypochlorous water to prevent a bias on the basis of olfactory cues with the apparatus. Detailed description of each behavioral test (neurological screen, neuromascular strength, rotarod test, open-field test, light/dark transition test, elevated plus-maze test, hot plate test, startle response/prepulse inhibition tests, social interaction test in a novel environment, sociability and social novelty preference test, social interaction test in home cage, T-maze test and contextual and cued fear conditioning) can be seen in Supplementary methods.

Statistical analysis

Tests for HWE and marker-trait association were evaluated by χ^2 test (SAS/Genetics, release 8.2, SAS Institute Japan Inc., Tokyo, Japan). Gene-wide significance of single-SNP test was estimated by permuting phenotype status to generate 10 000 data set of SNPs in each gene under null hypothesis of no association (49). Differences in relative expression between alleles (for luciferase assay) and genotypes (for real-time PCR and western blot) were evaluated by a two-tailed Student's *t*-test and one-way ANOVA, respectively (JMP5.1J, SAS Institute Japan Inc.). When a significant difference was obtained in ANOVA, *post hoc* comparison with the Dunnett test [with homozygous major alleles (G/G genotype) set as controls] was used to identify specific group differences. Also to check the effects of haplotypes on gene expression, haplotype trend regression test with permutation (10 000 times) was applied (Power Marker V3.25 by Jack Liu, www://powermarker.net/). In behavior analysis, statistical analysis was conducted by using STATVIEW (SAS Institute, Cary, NC). Data were analyzed by ANOVA or repeated-measures ANOVA. Values in graphs were expressed as mean ± SEM. The level of significance was set at 0.05.

FUNDING

This research was supported in part by Grants-in-Aid for Scientific Research from the Ministry of Education, Culture, Sports, Science and Technology of Japan (MEXT), a Grant-in-Aid for Creative Scientific Research from Japan Society for the Promotion of Science, the MEXT 21st Century Center of Excellence Program, a research grant for Nervous and Mental Disorders from the Ministry of Health, Labour and Welfare, a Grant-in-Aid for Scientific Research on Pathomechanisms of Brain Disorders from the MEXT (17025021), the Japan Health Sciences Foundation (Research on Health Sciences Focusing on Drug Innovation), the Core Research for Evolutional Science and Technology,

Grant-in-Aid for Scientific Research on Priority Areas—Integrative Brain Research (Shien)—from MEXT in Japan, Grant-in-Aid from Neuroinformatics Japan Center (NIJC), RIKEN and Grant-in-Aid from Institute for Bioinformatics Research and Development (BIRD) of Japan Science and Technology Agency (JST).

SUPPLEMENTARY MATERIAL

Supplementary Material is available at *HMG* Online.

Conflict of Interest statement: None declared.

REFERENCES

1. Shenton, M.E., Dickey, C.C., Frumin, M. and McCarley, R.W. (2001) A review of MRI findings in schizophrenia. *Schizophr. Res.*, **49**, 1–52.
2. Pantelis, C., Yucel, M., Wood, S.J., Velakoulis, D., Sun, D., Berger, G., Stuart, G.W., Yung, A., Phillips, L. and McGorry, P.D. (2005) Structural brain imaging evidence for multiple pathological processes at different stages of brain development in schizophrenia. *Schizophr. Bull.*, **31**, 672–696.
3. Stephan, K.E., Baldeweg, T. and Friston, K.J. (2006) Synaptic plasticity and dysconnection in schizophrenia. *Biol. Psychiatry*, **59**, 929–939.
4. Paul, L.K., Brown, W.S., Adolphs, R., Tyszka, J.M., Richards, L.J., Mukherjee, P. and Sherr, E.H. (2007) Agenesis of the corpus callosum: genetic, developmental and functional aspects of connectivity. *Nat. Rev. Neurosci.*, **8**, 287–299.
5. Frankle, W.G., Lerma, J. and Laruelle, M. (2003) The synaptic hypothesis of schizophrenia. *Neuron*, **39**, 205–216.
6. Stefansson, H., Sigurdsson, E., Steinthorsdottir, V., Bjornsdottir, S., Sigmundsson, T., Ghosh, S., Brynjolfsson, J., Gunnarsdottir, S., Ivarsson, O., Chou, T.T. *et al.* (2002) Neuregulin 1 and susceptibility to schizophrenia. *Am. J. Hum. Genet.*, **71**, 877–892.
7. Straub, R.E., Jiang, Y., MacLean, C.J., Ma, Y., Webb, B.T., Myakishev, M.V., Harris-Kerr, C., Wormley, B., Sadek, H., Kadambi, B. *et al.* (2002) Genetic variation in the 6p22.3 gene DTNBP1, the human ortholog of the mouse dysbindin gene, is associated with schizophrenia. *Am. J. Hum. Genet.*, **71**, 337–348.
8. Chumakov, I., Blumenfeld, M., Guerassimenko, O., Cavarec, L., Palicio, M., Abderrahim, H., Bougueleret, L., Barry, C., Tanaka, H., La Rosa, P. *et al.* (2002) Genetic and physiological data implicating the new human gene G72 and the gene for D-amino acid oxidase in schizophrenia. *Proc. Natl. Acad. Sci. USA*, **99**, 13675–13680.
9. Egan, M.F., Goldberg, T.E., Kolachana, B.S., Callicott, J.H., Mazzanti, C.M., Straub, R.E., Goldman, D. and Weinberger, D.R. (2001) Effect of COMT Val108/158 Met genotype on frontal lobe function and risk for schizophrenia. *Proc. Natl. Acad. Sci. USA*, **98**, 6917–6922.
10. Bilder, R.M., Volavka, J., Czobor, P., Malhotra, A.K., Kennedy, J.L., Ni, X., Goldman, R.S., Hoptman, M.J., Sheitman, B., Lindenmayer, J.P. *et al.* (2002) Neurocognitive correlates of the COMT Val(158)Met polymorphism in chronic schizophrenia. *Biol. Psychiatry*, **52**, 701–707.
11. Shifman, S., Bronstein, M., Sternfeld, M., Pisante-Shalom, A., Lev-Lehman, E., Weizman, A., Reznik, I., Spivak, B., Grisaru, N., Karp, L. *et al.* (2002) A highly significant association between a COMT haplotype and schizophrenia. *Am. J. Hum. Genet.*, **71**, 1296–1302.
12. Craddock, N., O'Donovan, M.C. and Owen, M.J. (2005) The genetics of schizophrenia and bipolar disorder: dissecting psychosis. *J. Med. Genet.*, **42**, 193–204.
13. Harrison, P.J. and Weinberger, D.R. (2005) Schizophrenia genes, gene expression, and neuropathology: on the matter of their convergence. *Mol. Psychiatry*, **10**, 40–68.
14. Millar, J.K., Wilson-Annan, J.C., Anderson, S., Christie, S., Taylor, M.S., Semple, C.A., Devon, R.S., Clair, D.M., Muir, W.J., Blackwood, D.H. *et al.* (2000) Disruption of two novel genes by a translocation co-segregating with schizophrenia. *Hum. Mol. Genet.*, **9**, 1415–1423.
15. Blackwood, D.H., Fordyce, A., Walker, M.T., St Clair, D.M., Porteous, D.J. and Muir, W.J. (2001) Schizophrenia and affective disorders—cosegregation with a translocation at chromosome 1q42 that directly disrupts brain-expressed genes: clinical and P300 findings in a family. *Am. J. Hum. Genet.*, **69**, 428–433.
16. Millar, J.K., Pickard, B.S., Mackie, S., James, R., Christie, S., Buchanan, S.R., Malloy, M.P., Chubb, J.E., Huston, E., Baillie, G.S. *et al.* (2005) DISC1 and PDE4B are interacting genetic factors in schizophrenia that regulate cAMP signaling. *Science*, **310**, 1187–1191.
17. Ozeki, Y., Tomoda, T., Kleiderlein, J., Kamiya, A., Bord, L., Fujii, K., Okawa, M., Yamada, N., Hatten, M.E., Snyder, S.H. *et al.* (2003) Disrupted-in-Schizophrenia-1 (DISC-1): mutant truncation prevents binding to NudE-like (NUDEL) and inhibits neurite outgrowth. *Proc. Natl. Acad. Sci. USA*, **100**, 289–294.
18. Millar, J.K., Christie, S. and Porteous, D.J. (2003) Yeast two-hybrid screens implicate DISC1 in brain development and function. *Biochem. Biophys. Res. Commun.*, **311**, 1019–1025.
19. Morris, J.A., Kandpal, G., Ma, L. and Austin, C.P. (2003) DISC1 (Disrupted-In-Schizophrenia 1) is a centrosome-associated protein that interacts with MAP1A, MIPT3, ATF4/5 and NUDEL: regulation and loss of interaction with mutation. *Hum. Mol. Genet.*, **12**, 1591–1608.
20. Brandon, N.J., Handford, E.J., Schurov, I., Rain, J.C., Pelling, M., Duran-Jimeniz, B., Camargo, L.M., Oliver, K.R., Beher, D., Shearman, M.S. *et al.* (2004) Disrupted in Schizophrenia 1 and Nudel form a neurodevelopmentally regulated protein complex: implications for schizophrenia and other major neurological disorders. *Mol. Cell. Neurosci.*, **25**, 42–55.
21. Miyoshi, K., Honda, A., Baba, K., Taniguchi, M., Oono, K., Fujita, T., Kuroda, S., Katayama, T. and Tohyama, M. (2003) Disrupted-in-Schizophrenia 1, a candidate gene for schizophrenia, participates in neurite outgrowth. *Mol. Psychiatry*, **8**, 685–694.
22. Taya, S., Shinoda, T., Tsuboi, D., Asaki, J., Nagai, K., Hikita, T., Kuroda, S., Kuroda, K., Shimizu, M., Hirotsune, S. *et al.* (2007) DISC1 regulates the transport of the NUDEL/LIS1/14-3-3 complex through Kinesin-1. *J. Neurosci*, **27**, 15–26.
23. Shinoda, T., Taya, S., Tsuboi, D., Hikita, T., Matsuzawa, R., Kuroda, S., Iwamatsu, A. and Kaibuchi, K. (2007) DISC1 regulates neurotrophin-induced axon elongation via interaction with Grb2. *J. Neurosci*, **27**, 4–14.
24. Muslin, A.J., Tanner, J.W., Allen, P.M. and Shaw, A.S. (1996) Interaction of 14-3-3 with signaling proteins is mediated by the recognition of phosphoserine. *Cell*, **84**, 889–897.
25. Muslin, A.J. and Xing, H. (2000) 14-3-3 proteins: regulation of subcellular localization by molecular interference. *Cell Signal.*, **12**, 703–709.
26. Yaffe, M.B., Rittinger, K., Volinia, S., Caron, P.R., Aitken, A., Leffers, H., Gamblin, S.J., Smerdon, S.J. and Cantley, L.C. (1997) The structural basis for 14-3-3:phosphopeptide binding specificity. *Cell*, **91**, 961–971.
27. Fu, H., Subramanian, R.R. and Masters, S.C. (2000) 14-3-3 proteins: structure, function, and regulation. *Annu. Rev. Pharmacol. Toxicol.*, **40**, 617–647.
28. Tzivion, G. and Avruch, J. (2002) 14-3-3 proteins: active cofactors in cellular regulation by serine/threonine phosphorylation. *J. Biol. Chem.*, **277**, 3061–3064.
29. Toyo-oka, K., Shionoya, A., Gambello, M.J., Cardoso, C., Leventer, R., Ward, H.L., Ayala, R., Tsai, L.H., Dobyns, W., Ledbetter, D. *et al.* (2003) 14-3-3epsilon is important for neuronal migration by binding to NUDEL: a molecular explanation for Miller–Dieker syndrome. *Nat. Genet.*, **34**, 274–285.
30. Crawly (2000) *What's Wrong with My Mouse? Behavioral Phenotyping of Transgenic and Knockout Mice*, Wiley, New York.
31. Takao, K. and Miyakawa, T. (2006) Investigating gene-to-behavior pathways in psychiatric disorders: the use of a comprehensive behavioral test battery on genetically engineered mice. *Ann. N. Y. Acad. Sci.*, **1086**, 144–159.
32. Eckerman, D.A., Gordon, W.A., Edwards, J.D., MacPhail, R.C. and Gage, M.I. (1980) Effects of scopolamine, pentobarbital, and amphetamine on radial arm maze performance in the rat. *Pharmacol. Biochem. Behav.*, **12**, 595–602.
33. Okaichi, H. and Jarrard, L.E. (1982) Scopolamine impairs performance of a place and cue task in rats. *Behav. Neural. Biol.*, **35**, 319–325.
34. Yanagi, M., Shirakawa, O., Kitamura, N., Okamura, K., Sakurai, K., Nishiguchi, N., Hashimoto, T., Nushida, H., Ueno, Y., Kanbe, D. *et al.* (2005) Association of 14-3-3 epsilon gene haplotype with completed suicide in Japanese. *Hum. Mol. Genet.*, **50**, 210–216.

35. Harrison, P.J. (1999) The neuropathology of schizophrenia. A critical review of the data and their interpretation. *Brain*, **122** (Pt 4), 593–624.
36. Lewis, D.A. and Levitt, P. (2002) Schizophrenia as a disorder of neurodevelopment. *Annu. Rev. Neurosci.*, **25**, 409–432.
37. Kamiya, A., Kubo, K.I., Tomoda, T., Takaki, M., Youn, R., Ozeki, Y., Sawamura, N., Park, U., Kudo, C., Okawa, M. *et al.* (2005) A schizophrenia-associated mutation of DISC1 perturbs cerebral cortex development. *Nat. Cell Biol.*, **7**, 1167–1178.
38. Clapcote, S.J., Lipina, T.V., Millar, J.K., Mackie, S., Christie, S., Ogawa, F., Lerch, J.P., Trimble, K., Uchiyama, M., Sakuraba, Y. *et al.* (2007) Behavioral phenotypes of Disc1 missense mutations in mice. *Neuron*, **54**, 387–402.
39. Goldman-Rakic, P.S. (1994) Working memory dysfunction in schizophrenia. *J. Neuropsychiatry Clin. Neurosci.*, **6**, 348–357.
40. Elvevag, B. and Goldberg, T.E. (2000) Cognitive impairment in schizophrenia is the core of the disorder. *Crit. Rev. Neurobiol.*, **14**, 1–21.
41. Callicott, J.H., Egan, M.F., Mattay, V.S., Bertolino, A., Bone, A.D., Verchinksi, B. and Weinberger, D.R. (2003) Abnormal fMRI response of the dorsolateral prefrontal cortex in cognitively intact siblings of patients with schizophrenia. *Am J. Psychiatry*, **160**, 709–719.
42. Callicott, J.H., Straub, R.E., Pezawas, L., Egan, M.F., Mattay, V.S., Hariri, A.R., Verchinski, B.A., Meyer-Lindenberg, A., Balkissoon, R., Kolachana, B. *et al.* (2005) Variation in DISC1 affects hippocampal structure and function and increases risk for schizophrenia. *Proc. Natl. Acad. Sci. USA*, **102**, 8627–8632.
43. Braff, D.L. and Geyer, M.A. (1990) Sensorimotor gating and schizophrenia. Human and animal model studies. *Arch. Gen. Psychiatry*, **47**, 181–188.
44. Duan, X., Chang, J.H., Ge, S., Faulkner, R.L., Kim, J.Y., Kitabatake, Y., Liu, X.B., Yang, C.H., Jordan, J.D., Ma, D.K. *et al.* (2007) Disrupted-in-Schizophrenia 1 regulates integration of newly generated neurons in the adult brain. *Cell*, **130**, 1146–1158.
45. Darling, D.L., Yingling, J. and Wynshaw-Boris, A. (2005) Role of 14-3-3 proteins in eukaryotic signaling and development. *Curr. Top. Dev. Biol.*, **68**, 281–315.
46. Collins, M.O., Husi, H., Yu, L., Brandon, J.M., Anderson, C.N., Blackstock, W.P., Choudhary, J.S. and Grant, S.G. (2006) Molecular characterization and comparison of the components and multiprotein complexes in the postsynaptic proteome. *J. Neurochem.*, **97** (Suppl 1), 16–23.
47. de Bakker, P.I., Yelensky, R., Pe'er, I., Gabriel, S.B., Daly, M.J. and Altshuler, D. (2005) Efficiency and power in genetic association studies. *Nat. Genet.*, **37**, 1217–1223.
48. Ikeda, M., Iwata, N., Suzuki, T., Kitajima, T., Yamanouchi, Y., Kinoshita, Y., Inada, T., Ujike, H. and Ozaki, N. (2005) Association analysis of chromosome 5 GABAA receptor cluster in Japanese schizophrenia patients. *Biol. Psychiatry*, **58**, 440–445.
49. Dudbridge, F. (2003) Pedigree disequilibrium tests for multilocus haplotypes. *Genet Epidemiol*, **25**, 115–121.

BMC Psychiatry

BioMed Central

Research article

Open Access

Association study of polymorphisms in the neutral amino acid transporter genes *SLC1A4*, *SLC1A5* and the glycine transporter genes *SLC6A5*, *SLC6A9* with schizophrenia

Xiangdong Deng[1], Noriaki Sagata[1], Naoko Takeuchi[1], Masami Tanaka[1], Hideaki Ninomiya[2], Nakao Iwata[3], Norio Ozaki[4], Hiroki Shibata*[1] and Yasuyuki Fukumaki[1]

Address: [1]Division of Human Molecular Genetics, Research Center for Genetic Information, Medical Institute of Bioregulation, Kyushu University, Fukuoka 812-8582, Japan, [2]Fukuoka Prefectural Dazaifu Hospital Psychiatric Center, Dazaifu, Fukuoka, Japan, [3]Department of Psychiatry, Fujita Health University School of Medicine, Toyoake, Aichi 470-1192, Japan and [4]Department of Psychiatry, Graduate School of Medicine, Nagoya University, Nagoya, Japan

Email: Xiangdong Deng - luckbird@gen.kyushu-u.ac.jp; Noriaki Sagata - asagata@gen.kyushu-u.ac.jp; Naoko Takeuchi - naoko.takeuchi@roche.com; Masami Tanaka - mtanaka@gen.kyushu-u.ac.jp; Hideaki Ninomiya - ninoh@d-med.pref.fukuoka.jp; Nakao Iwata - nakao@fujita-hu.ac.jp; Norio Ozaki - ozaki-n@med.nagoya-u.ac.jp; Hiroki Shibata* - hshibata@gen.kyushu-u.ac.jp; Yasuyuki Fukumaki - yfukumak@gen.kyushu-u.ac.jp

* Corresponding author

Published: 18 July 2008

BMC Psychiatry 2008, 8:58 doi:10.1186/1471-244X-8-58

Received: 12 November 2007
Accepted: 18 July 2008

This article is available from: http://www.biomedcentral.com/1471-244X/8/58

© 2008 Deng et al; licensee BioMed Central Ltd.
This is an Open Access article distributed under the terms of the Creative Commons Attribution License (http://creativecommons.org/licenses/by/2.0), which permits unrestricted use, distribution, and reproduction in any medium, provided the original work is properly cited.

Abstract

Background: Based on the glutamatergic dysfunction hypothesis for schizophrenia pathogenesis, we have been performing systematic association studies of schizophrenia with the genes involved in glutametergic transmission. We report here association studies of schizophrenia with *SLC1A4*, *SLC1A5* encoding neutral amino acid transporters ASCT1, ASCT2, and *SLC6A5*, *SLC6A9* encoding glycine transporters GLYT2, GLYT1, respectively.

Methods: We initially tested the association of 21 single nucleotide polymorphisms (SNPs) distributed in the four gene regions with schizophrenia using 100 Japanese cases-control pairs and examined allele, genotype and haplotype association with schizophrenia. The observed nominal significance were examined in the full-size samples (400 cases and 420 controls).

Results: We observed nominally significant single-marker associations with schizophrenia in SNP2 ($P = 0.021$) and SNP3 ($P = 0.029$) of *SLC1A4*, SNP1 ($P = 0.009$) and SNP2 ($P = 0.022$) of *SLC6A5*. We also observed nominally significant haplotype associations with schizophrenia in the combinations of SNP2-SNP7 ($P = 0.037$) of *SLC1A4* and SNP1-SNP4 ($P = 0.043$) of *SLC6A5*. We examined all of the nominal significance in the Full-size Sample Set, except one haplotype with insufficient LD. The significant association of SNP1 of *SLC6A5* with schizophrenia was confirmed in the Full-size Sample Set ($P = 0.018$).

Conclusion: We concluded that at least one susceptibility locus for schizophrenia may be located within or nearby *SLC6A5*, whereas *SLC1A4*, *SLC1A5* and *SLC6A9* are unlikely to be major susceptibility genes for schizophrenia in the Japanese population.

Background

Schizophrenia is a devastating mental disorder that affects about 1% of worldwide populations [1], and genetic factors are known to play a crucial role in its pathogenesis [2]. The successful treatment with dopamine antagonists on the positive symptomatology of the disease suggests a crucial role of dopamine in the pathophysiology of schizophrenia. However, due to the poor effects of dopamine antagonists against the negative and cognitive symptoms of schizophrenia, other neurotransmitter systems than dopamine, such as glutamate are suggested to be involved in the pathogenesis of schizophrenia. Based on the fact that phencyclidine (PCP), the antagonist of N-methyl-D-aspartate (NMDA) glutamate receptor, induces schizophreniform psychosis, a glutamatergic dysfunction hypothesis has been proposed for the pathogenesis of schizophrenia [3-5]. This hypothesis has been supported by recent multiple reports of significant association of schizophrenia with glutamate receptor genes and with the genes related to glutamatergic transmission [Review, [6,7]]. The dopamine and glutamate hypothesis of schizophrenia are not independent, and in fact, glutamate-dopamine interaction has been supported by many preclinical and clinical findings [Review, [8]].

Other synaptic elements related to glutamate, such as transporters, also potentially affect glutamatergic neurotransmission. Excitatory amino acid transporters (EAATs) maintain extracellular glutamate concentrations within physiological levels by reuptaking synaptically released glutamate. Abnormalities of mRNA expression of EAATs were reported in the thalamus, prefrontal cortex, parahippocampal gyrus and striatum in schizophrenia [9-12]. Recently, we have reported the positive association of *SLC1A2* and *SLC1A6*, the genes encoding EAAT2 and EAAT4, respectively with schizophrenia [13,14], providing support for the potential important roles of EAATs in schizophrenia.

Neutral amino acid transporters (ASCTs), which transport neutral amino acid (alanine, serine, cysteine and threonin) were identified based on nucleotide sequence homology to the EAATs [15,16]. The amino acid identity between EAATs and ASCTs is 40–44%. The functions of ASCTs in glutamate transmission have also been reported. ASCT1 not only mediates the efflux of glutamate from the neuron into the synaptic junction via Calcium-independent release, but also mediates the efflux of L-serine from glial cells and its uptake by neurons [17-19]. L-serine is used for syntheses of various biomolecules, including the co-agonists at NMDA glutamate receptor, D-serine and glycine. ASCT2 appears to play an important role in the glutamine-glutamate cycle between neurons and glia by facilitation the efflux of glutamine from glial cells [20]. Recently, Weis et al. reported significant decrease in ASCT1 immunoreactivity in the cingulate cortex, white matter, and striking loss of ASCT1 immunoreactivity in the hippocampus in schizophrenia. [21].

Glycine acts as an obligatory co-agonist at NMDA glutamate receptor to promote NMDA receptor function. In the central system, the actions of glycine are terminated by its rapid uptake into the nerve terminal and adjacent glial cells via high-affinity glycine transporters (GLYTs) [22]. Therefore, increasing synaptic level of glycine by inhibiton of its uptake could lead to enhance the activation of NMDA receptor. Both preclinical and clinical evidence have provided support for the utility of this modulatory approach, as well as the potential therapeutic value of GLYT1 inhibitors in the treatment of schizophrenia [Review, [23]]. Therefore the ASCTs and GLYTs genes are strong candidates for schizophrenia, as well as glutamate receptor and glutamate transporter genes.

In this study we report association studies of schizophrenia with total 21 SNPs distributed in genes *SLC1A4*, *SLC1A5*, *SLC6A5* and *SLC6A9* that encoding the neutral amino acid transporters ASCT1, ASCT2 and the glycine transporters GLYT2, GLYT1, respectively. SNPs were selected to cover the entire gene regions by linkage disequilibria (LD). To enhance the detection power of the study, we also examined the haplotype associations with the disease.

Methods

Human subjects

Blood samples were obtained from unrelated Japanese individuals who had provided written informed consent. We used 400 cases (mean age 47.2; 44.8% female) recruited from hospitals in Kyushu and Aichi areas and 420 unrelated controls (mean age 43.6; 44.0% female) recruited from the Kyushu and Aichi areas. We initially tested the association of the genes with schizophrenia using the Screening Sample Set: 100 out of 400 cases (mean age 49.5; 44.0% female) and 100 out of 420 controls (mean age 51.2; 44.0% female) recruited from the Kyushu area. All patients were diagnosed by the Diagnostic and Statistical Manual of Mental Disorders (DSM)-IV criteria [24]. The patients are all consecutive inpatients. The schizophrenia diagnoses were confirmed by several psychiatrists. We used another 16 healthy Japanese samples to test the frequencies of the candidate SNPs selected from the database. This study was approved by the Ethics Committee of Kyushu University, Faculty of Medicine. DNA samples were extracted from leukocytes by standard methods [25].

SNP selection

We retrieved the primary SNP information from the dbSNP database [26]. Assuming the same size of the half

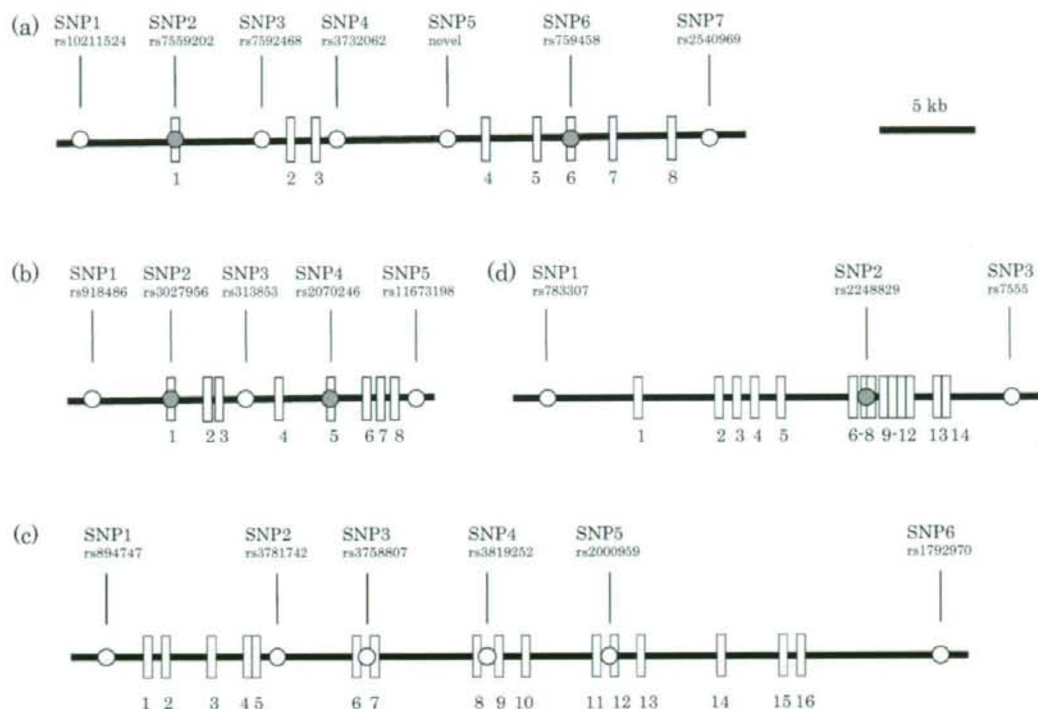

Figure 1

Genomic organizations of each gene and locations of the SNPs. (a) *SLC1A4* spans over 34 kb and is composed of 8 exons. (b) *SLC1A5* spans over 14 kb and is composed of 8 exons. (c) *SLC6A5* spans over 55.6 kb and is composed of 16 exons. (d) *SLC6A9* spans over 19.8 kb and is composed of 14 exons. Exons are shown as vertical bars with exon numbers. SNPs we analyzed are indicated by circles. Exonic SNPs are indicated by filled circles.

length of LD (60 kb) as reported in Caucasians [27], we initially intended to select common SNPs every 30 kb in the three genes including all of the exonic SNPs. We tested the frequencies of the candidate SNPs, in the 16 healthy Japanese samples by the direct sequencing method. Out of them, common SNPs with minor allele frequencies over 10% were selected for further analyses. The SNPs in which significant deviation from Hardy-Weinberg equilibrium (HWE) observed in the 100 control samples were replaced by another SNP nearby. Since LD gaps ($D' < 0.3$) were observed in the initial SNP set after the LD analyses described below, we selected additional SNPs to fill the LD gaps.

We finally selected the following 21 common SNPs distributed across the gene regions for further analyses: 7 of *SLC1A4*, SNP1, rs10211524; SNP2, rs7559202; SNP3, rs7592468; SNP4, rs3732062; SNP5, novel SNP in intron 3 (located on 33bp 5' to SNP rs7583682); SNP6, rs759458; SNP7, rs2540969, 5 of *SLC1A5*, SNP1, rs918486; SNP2, rs3027956; SNP3, rs313853; SNP4, rs2070246; SNP5, rs11673198, 6 of *SLC6A5*, SNP1, rs894747; SNP2, rs3781742; SNP3, rs3758807; SNP4, rs3819252; SNP5, rs2000959, SNP6, rs1792970 and 3 of *SLC6A9*, SNP1, rs783307; SNP2, rs2248829; SNP3, rs7555. The locations of the 21 SNPs are shown in Figure 1.

Genotyping

The 21 SNPs were amplified as individual fragments by PCR as previously described [14]. The nucleotide sequences of each primer, PCR conditions and genotyping methods for each SNP are shown in Additional File 1. Because of the GC rich sequences of *SLC1A5* region, we used Fail Safe PCR system (Epicentre Technologies) to optimize the PCR conditions when amplified SNP1, SNP2

and SNP5 of *SLC1A5*. We genotyped samples for SNP5 of *SLC6A5* by polymerase chain reaction/restriction fragment length polymorphism (PCR-RFLP) and the other 20 SNPs by direct sequencing, as previously described [28]. The raw data of direct sequencing were compiled on PolyPhred [29] and/or Mutation Surveyor (SoftGenetics LLC).

Statistical analyses
To control genotyping errors, Hardy-Weinberg equilibrium (HWE) for the genotype frequencies was checked in the control samples by the χ^2-test (d.f. = 1). We evaluated the statistical differences in genotype and allele frequencies between cases and controls by Fisher's exact probability test. The magnitude of linkage disequilibrium (LD) was evaluated by caculating D' using the haplotype frequencies estimated by the EH program, version 1.14 [30], and D' is represented graphically using the software Graphical Overview of Linkage Disequilibrium (GOLD) [31]. Statistical analysis of the haplotype association was carried out as previously described [32]. The significance level for all statistical tests was 0.05. We adjusted the *P* values of association studies for multiplicity using a false discovery rate (FDR) controlling procedure [33].

Results
Genotyping and SNP association analyses
We selected total 21 SNPs at an average interval of 7.8 kb for *SLC1A4*, 4.5 kb for *SLC1A5*, 14.2 kb for *SLC6A5* and 18.9 kb for *SLC6A9* to cover each entire gene region with LD. Since the average allele frequencies of the SNPs are 0.35, 0.30, 0.37 and 0.30 respectively, the expected detection powers for *SLC1A4*, *SLC1A5*, *SLC6A5* and *SLC6A9* are 0.84, 0.82, 0.84 and 0.82, respectively, under the multiplicative model with genotype relative risk = 1.8 [34]. Considering the high expected detection powers, we initially tested the single-marker association of the 21 SNPs with schizophrenia using the Screening Sample Set (100 cases and 100 controls) by the method described above, and investigated the association in the Full-size Sample Set (400 cases and 420 controls) only for the SNPs that showed significant single-marker or haplotype association with schizophrenia in the Screening Sample Set.

Table 1 shows the results of genotype and allele frequencies of SNPs in 100 case and 100 control samples. No significant deviation from HWE in control samples was observed in these SNPs (data not shown). We observed significant associations with schizophrenia in allele frequencies of SNP2 and SNP3 of *SLC1A4* ($P = 0.021$, $P = 0.029$, respectively), and in genotype frequencies of SNP1 and SNP2 of *SLC6A5* ($P = 0.009$, $P = 0.022$, respectively), although none of them survived after controlling the FDR at level 0.05 (n = 7 for *SLC1A4* and n = 6 for *SLC6A5*).

Pairwise linkage disequilibrium and haplotype association analyses
We compared the magnitude of LD for all possible pairs of the SNPs in each gene region in controls and cases by calculating D' (Figure 2). No essential difference was shown in the LD pattern of any genes between cases and controls. Strong or modest LD ($D' > 0.3$) were observed in all combinations of adjacent SNPs in *SLC1A5* and *SLC6A9* regions. Whereas in each small subregion of the other two gene regions, LD drops abruptly: SNP4-SNP5 of *SLC1A4* and SNP2-SNP3 of *SLC6A5* ($D' = 0.061$ and $D' = 0.125$, respectively).

We constructed pairwise haplotypes for all of the possible SNP pairs (Table 2). We observed significant associations with schizophrenia in combinations of SNP2-SNP7 of *SLC1A4* ($P = 0.037$) and SNP1-SNP4 of *SLC6A5* ($P = 0.043$). However, neither of them survived after controlling the FDR at level 0.05.

Association analyses using the Full-size Sample Set
Since nominally significant single-marker and haplotype associations with schizophrenia were observed in the Screening Sample Set, we genotyped the Full-size Sample Set for the SNPs involved in the significance, SNP2, SNP3 of *SLC1A4* and SNP1, SNP2, SNP4 of *SLC6A5* to examine these significant associations in the Full-size Sample Set. We excluded SNP7 of *SLC1A4* from further analyses in the Full-size Sample Set because of the insufficient D' observed in the combination of SNP2-SNP7 in both cases and controls ($D' = 0.064$ and $D' = 0.171$, respectively). The genotype and allele frequencies of each SNP in the Full-size Sample Set are shown in the Additional File 2. The significant association of SNP1 of *SLC6A5* with schizophrenia was confirmed in both genotype and allele frequencies in the Full-size Sample Set ($P = 0.032$, $P = 0.018$, respectively). We failed to detect other single-marker associations (*P* value range 0.065 – 0.355) and the haplotype association ($P = 0.088$) observed in the initial screening.

Discussion
SLC1A4, *SLC1A5*, *SLC6A5* and *SLC6A9* were located on chromosome 2p13-15, 19q13.3, 11p15.2-p15.1, and 1p33, respectively. Suggestive evidence for linkage of chromosome 2p14-p13, where *SLC1A4* is located, with schizophrenia has been reported in schizophrenia families from Palau and Ireland [35,36]. However, the subsequent mutation screening failed to find any sequence polymorphism segregated with the illness in the *SLC1A4* region of the Palauan families [37]. In addition, negative association of *SLC1A4* with schizophrenia was reported in the German population [38]. There has been no linkage with schizophrenia reported to the chromosome regions where *SLC1A5*, *SLC6A5* or *SLC6A9* are located [36]. Moreover, exclusion of linkage between schizophrenia and

Table 1: Genotype and allele frequencies of SNPs in each gene in the Screening Sample Set (100 cases and 100 controls)

Genes	Polymorphism	Genotype count			P^{*}	Allele frequency (%)		P^{**}
SLC1A4	SNP1	A/A	A/G	G/G		A	G	
	Schizophrenics	71	25	4	0.296	83.5	16.5	0.251
	Controls	61	35	4		78.5	21.5	
	SNP2	C/C	C/G	G/G		C	G	
	Schizophrenics	12	35	53	0.090	29.5	70.5	0.021
	Controls	6	27	67		19.5	80.5	
	SNP3	A/A	A/G	G/G		A	G	
	Schizophrenics	53	35	12	0.117	70.5	29.5	0.029
	Controls	67	26	7		80	20	
	SNP4	A/A	A/G	G/G		A	G	
	Schizophrenics	25	45	30	0.315	47.5	52.5	0.547
	Controls	17	54	29		44	56	
	SNP5	C/C	C/T	T/T		C	T	
	Schizophrenics	64	33	3	0.778	80.5	19.5	0.605
	Controls	68	30	2		83	17	
	SNP6	A/A	A/G	G/G		A	G	
	Schizophrenics	1	22	77	0.096	12	88	0.123
	Controls	7	22	71		18	82	
	SNP7	C/C	C/T	T/T		C	T	
	Schizophrenics	23	42	35	0.194	44	56	0.316
	Controls	23	53	24		49.5	50.5	
SLC1A5	SNP1	A/A	A/G	G/G		A	G	
	Schizophrenics	63	26	11	0.172	76.0	24.0	0.813
	Controls	60	35	5		77.5	22.5	
	SNP2	C/C	C/G	G/G		C	G	
	Schizophrenics	36	45	19	0.964	58.5	41.5	0.839
	Controls	37	46	17		60.0	40.0	
	SNP3	C/C	C/T	T/T		C	T	
	Schizophrenics	11	34	55	0.816	28.0	72.0	> 0.999
	Controls	9	37	54		27.5	72.5	
	SNP4	C/C	C/T	T/T		C	T	
	Schizophrenics	51	40	9	0.819	71.0	29.0	> 0.999
	Controls	52	37	11		70.5	29.5	
	SNP5	C/C	C/T	T/T		C	T	
	Schizophrenics	51	41	8	0.775	71.5	28.5	1.000
	Controls	53	37	10		71.5	28.5	
SLC6A5	SNP1	A/A	A/G	G/G		A	G	
	Schizophrenics	17	65	18	0.009	49.5	50.5	0.109
	Controls	35	46	19		58.0	42.0	
	SNP2	C/C	C/T	T/T		C	T	
	Schizophrenics	20	64	16	0.022	52.0	48.0	0.107
	Controls	37	47	16		60.5	39.5	
	SNP3	C/C	C/T	T/T		C	T	
	Schizophrenics	50	40	10	0.757	70.0	30.0	0.666

Table 1: Genotype and allele frequencies of SNPs in each gene in the Screening Sample Set (100 cases and 100 controls) *(Continued)*

	Controls	45	45	10		67.5	32.5	
	SNP4	A/A	A/G	G/G		A	G	
	Schizophrenics	6	40	54	0.332	26.0	74.0	0.375
	Controls	12	37	51		30.5	69.5	
	SNP5	C/C	C/G	G/G		C	G	
	Schizophrenics	39	48	13	0.882	63.0	37.0	> 0.999
	Controls	40	45	15		62.5	37.5	
	SNP6	C/C	C/G	G/G		C	G	
	Schizophrenics	10	43	47	0.783	31.5	68.5	0.595
	Controls	13	43	44		34.5	65.5	
SLC6A9	SNP1	C/C	C/T	T/T		C	T	
	Schizophrenics	32	48	20	0.987	56.0	44.0	> 0.999
	Controls	31	49	20		55.5	44.5	
	SNP2	A/A	A/G	G/G		A	G	
	Schizophrenics	7	32	61	0.988	23.0	77.0	> 0.999
	Controls	7	31	62		22.5	77.5	
	SNP3	A/A	A/G	G/G		A	G	
	Schizophrenics	6	31	63	0.830	21.5	78.5	0.720
	Controls	6	35	59		23.5	76.5	

*Fisher's exact probability tests, case vs. control (2 × 3).
**Fisher's exact probability tests, case vs. control (2 × 2).

SLC1A5 in 23 English and Icelandic schizophrenia families was reported [39]. Recently, negative associations of schizophrenia with polymorphisms in *SLC6A9* and *SLC6A5* were reported in the Chinese and the German population, respectively [40,41]. We investigated the association of *SLC1A4*, *SLC1A5*, *SLC6A5* and *SLC6A9* genes with schizophrenia in the Japanese population by analysing total 21 common SNPs.

Since the frequencies of genotyped SNPs are over 0.3, the expected detection powers of the four genes are over 0.80, assuming the genotype relative risk of 1.8. However, assuming lower genotype relative risk of 1.5 or 1.3, the expected detection powers for the four genes dropped to 0.50 – 0.53 or 0.24 – 0.25, respectively. Consequently, the negative finding for genes and SNPs excluded from the analyses using the Full-size Sample Set in this study may be due to type II error at lower relative risks, and they need to be investigated further in an enlarged sample size.

Out of the 21 SNPs analyzed, two within *SLC1A4*, (SNP4 and SNP6, 330 cases and 319 controls) and one within *SLC6A5*, (SNP5, 328 cases and 307 controls) have recently been reported to show no association with schizophrenia in the German population [38,41]. We also observed no association of these SNPs with the disease in our Screening Sample Set. The SNP1 in *SLC6A5* of which we observed a significant association with the disease, was not included in the report mentioned above.

In LD analysis of the initial screening of the 21 SNPs distributed in the four genes, modest LD ($D' > 0.3$) was observed in all combinations of adjacent SNPs in controls except for the combinations of SNP4-SNP5 of *SLC1A4* and SNP2-SNP3 of *SLC6A5*, suggesting recombination hot spots in the two regions (6.6 kb and 7.6 kb, respectively) (Figure 2). We compared the LD structure to the publically open database, HapMap [42]. The LD gap we observed in the *SLC6A5* region was not observed in the HapMap LD structure from either Japanese or Chinese population data ($D' = 0.817$ and $D' = 1$, respectively). The other LD gap, which was observed in the *SLC1A4* region, failed to be compared due to the absence of the novel SNP we found.

We observed significant single-marker associations in SNP2 and SNP3 of *SLC1A4* in the Screening Sample Set. However, we failed to confirm these findings in the Full-size Sample Set. We attribute to type I error due to the small sample size used in the Screening Sample Set. On the other hand, the single-marker association of SNP1 (rs894747) in *SLC6A5* region, although it does not show the significant association with the disease in the independent 300 case and 320 control samples (0.092), it does show the significant association in the Full-size Sam-

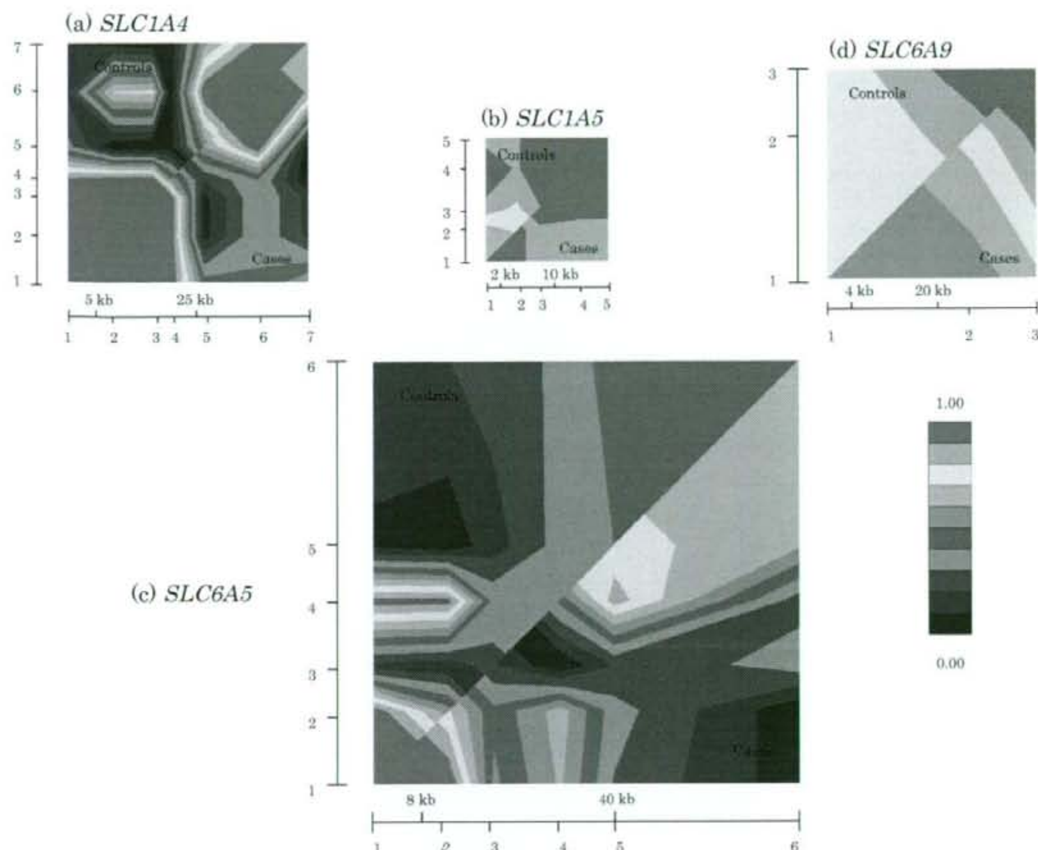

Figure 2 Pairwise LD analyses using GOLD for control (upper diagonal) and case (lower diagonal) haplotypes of each gene

Pairwise LD analyses using GOLD for control (upper diagonal) and case (lower diagonal) haplotypes of each gene. The relative location of markers used to construct the haplotypes is represented on the horizontal and vertical axes, which is more clearly depicted in Figure. 1. LD measure, *D'*, is graphically represented adjacent to each GOLD plot (red and dark blue are opposite ends of the scale).

ple Set ($P = 0.018$). We consider that the nonsignificant result observed in the enlarged samples may be due to the small sample size. SNP1 is located in the intergenic region, 2,355-bp upstream from the transcription start site. In the negative association report of *SLC6A5* in German population described above, four SNPs and one short-tandem-repeat distributed in intron 1~intron 11, but no SNP located in the upstream region were analysed [39]. In our Full-size Sample Set, the G allele was more frequently observed in schizophrenics (44.4%) than in controls (38.6%). Therefore, the G allele may be in LD with a risk allele for schizophrenia (odds ratio, 1.27; 95% confidence interval, 1.04~1.55). We conclude that at least one susceptibility locus for schizophrenia is located within or nearby *SLC6A5*, whereas *SLC1A4 SLC1A5* and *SLC6A9* are unlikely to be major susceptibility genes for schizophrenia in the Japanese population. No potential regulatory elements were previously identified in the region where SNP1 is located [43]. It is necessary to search for functional SNPs in the haplotype block where SNP1 is located. A copy number variation (CNV) has been reported in the European population on the chromosome 11p15.1, containing exon 15 of *SLC6A5* [44]. None of the 6 SNPs we genotyped is located within the CNV. Although the fre-

Table 2: Association analyses of pairwise haplotypes of SNPs in the genes

		SNP1	SNP2	SNP3	SNP4	SNP5	SNP6
SLC1A4	SNP2	0.118					
	SNP3	0.147	0.082				
	SNP4	0.178	0.096	0.132			
	SNP5	0.581	0.114	0.147	0.615		
	SNP6	0.079	0.050	0.062	0.239	0.401	
	SNP7	0.118	0.037	0.057	0.646	0.144	0.377
SLC1A5	SNP2	0.291					
	SNP3	0.978	0.845				
	SNP4	0.720	0.936	0.999			
	SNP5	0.984	0.944	1.000	0.483		
SLC6A5	SNP2	0.209					
	SNP3	0.241	0.178				
	SNP4	0.043	0.080	0.424			
	SNP5	0.243	0.160	0.936	0.083		
	SNP6	0.348	0.275	0.932	0.724	0.806	
SLC6A9	SNP2	0.854					
	SNP3	0.940	0.362				

P values by the two-tailed χ^2-test (d.f. = 3).

quency of the CNV in the Japanese population is unknown, SNP1 may be associated with the variant devoid of exon 15, which is a strong candidate of the susceptible allele. Therefore, it is necessary to test the association of the CNV with schizophrenia in Japanese sample sets. The positive association observed in *SLC6A5* also needs to be validated in different ethnic populations.

Conclusion

We conclude that at least one susceptibility locus for schizophrenia is located within or nearby *SLC6A5*, whereas *SLC1A4 SLC1A5* and *SLC6A9* are unlikely to be major susceptibility genes for schizophrenia in the Japanese population.

Competing interests

The authors declare that they have no competing interests.

Authors' contributions

XD carried out a portion of genotyping, statistical analyses and drafted the manuscript; NS, NT, MT carried out a portion of genotyping and statistical analyses; HN, NI and NO participated in collecting specimens and clinical data; HS participated in design of this study and statistical analyses; YF conceived of the study and participated in its design and coordination. All authors read and approved the final manuscript.

Additional material

Additional file 1

PCR primers for genotyping of SNPs in the genes. The data provided the nucleotide sequences of primers, PCR conditions and genotyping methods for each SNP.
Click here for file
[http://www.biomedcentral.com/content/supplementary/1471-244X-8-58-S1.xls]

Additional file 2

Genotype and allele frequencies of SNPs in the Full-size Sample Set (400 cases and 420 controls). The data show genotype and allele frequencies of SNPs in the Full-size Sample Set.
Click here for file
[http://www.biomedcentral.com/content/supplementary/1471-244X-8-58-S2.xls]

Acknowledgements

We are grateful to all the medical staff involved in collecting specimens, especially to Dr. Nobutada Tashiro, the emeritus professor of Kyushu University Graduate School of Medical Science for the initial support to our project. This work was supported by KAKENHI (Grant-in-Aid for Scientific Research) on Priority Areas "Applied Genomics" and other grants from the Ministry of Education, Culture, Sports, Science and Technology of Japan and by The Naito Foundation Subsidy for Natural Science Researches.

References

1. Gottesman PE: **Schizophrenia genesis: The origins of madness.** New York: Freeman; 1995.
2. McGuffin P, Owen MJ, Farmer AE: **Genetic basis of schizophrenia.** *Lancet* 1995, **346**:678-682.
3. Luby ED, Cohen BD, Rosenbaum G, Gottlieb JS, Kelley R: **Study of a new schizophrenomimetic drug, sernyl.** *AMA Arch Neurol Psychiatry* 1959, **81(3)**:363-369.
4. Javitt DC, Zukin SR: **Recent advances in the phencyclidine model of schizophrenia.** *Am J Psychiatry* 1991, **148**:1301-1308.
5. Mohn AR, Gainetdinov RR, Caron MG, Koller BH: **Mice with reduced NMDA receptor expression display behaviors related to schizophrenia.** *Cell* 1999, **98**:427-436.
6. Harrison PJ, Weinberger DR: **Schizophrenia genes, gene expression, and neuropathology: on the matter of their convergence.** *Mol Psychiatry* 2005, **10**:40-68.
7. Riley B, Kendler KS: **Molecular genetic studies of schizophrenia.** *Eur J Hum Genet* 2006, **14**:669-80.
8. de Bartolomeis A, Fiore G, Iasevoli F: **Dopamine-glutamate interaction and antipsychotics mechanism of action: implication for new pharmacological strategies in psychosis.** *Curr Pharm Des* 2005, **11**:3561-94.
9. Smith RE, Haroutunian V, Davis KL, Meador-Woodruff JH: **Expression of excitatory amino acid transporter transcripts in the thalamus of subjects with schizophrenia.** *Am J Psychiat* 2001, **158**:1393-1399.
10. Matute C, Melone M, Vallejo-Illarramendi A, Conti F: **Increased expression of the astrocytic glutamate transporter GLT-1 in the prefrontal cortex of schizophrenics.** *Glia* 2005, **49**:451-455.
11. Ohnuma T, Tessler S, Arai H, Faull RL, McKenna PJ, Emson PC: **Gene expression of metabotropic glutamate receptor 5 and excitatory amino acid transporter 2 in the schizophrenic hippocampus.** *Mol Brain Res* 2000, **85**:24-31.
12. McCullumsmith RE, Meador-Woodruff JH: **Striatal excitatory amino acid transporter transcript expression in schizophrenia, bipolar disorder, and major depressive disorder.** *Neuropsychopharmacology* 2002, **26**:368-375.
13. Deng X, Shibata H, Ninomiya H, Tashiro N, Iwata N, Ozaki N, Fukumaki Y: **Association study of polymorphisms in the excitatory**

amino acid transporter 2 gene (*SLC1A2*) with schizophrenia. *BMC Psychiatry* 2004, **4**:21.
14. Deng X, Shibata H, Takeuchi N, Rachi S, Sakai M, Ninomiya H, Iwata N, Ozaki N, Fukumaki Y: **Association study of polymorphisms in the glutamate transporter genes *SLC1A1*, *SLC1A3*, and *SLC1A6* with schizophrenia.** *Am J Med Genet B Neuropsychiatr Genet* 2007, **144B(3)**:271-8.
15. Arriza JL, Kavanaugh MP, Fairman WA, Wu YN, Murdoch GH, North RA, Amara SG: **Cloning and expression of a human neutral amino acid transporter with structural similarity to the glutamate transporter gene family.** *J Biol Chem* 1993, **268**:15329-15332.
16. Utsunomiya-Tate N, Endou H, Kanai Y: **Cloning and functional characterization of a system ASC-like Na+dependent neutral amino acid transporter.** *J Biol Chem* 1996, **271**:14883-14890.
17. Weiss MD, Derazi S, Kilberg MS, Anderson KJ: **Ontogeny and localization of the neutral amino acid transporter ASCT1 in rat brain.** *Brain Res Dev Brain Res* 2001, **130**:183-90.
18. Sakai K, Shimizu H, Koike T, Furuya S, Watanabe M: **Neutral amino acid transporter ASCT1 is preferentially expressed in L-Ser-synthetic/storing glial cells in the mouse brain with transient expression in developing capillaries.** *J Neurosci* 2003, **23**:550-60.
19. Yamamoto T, Nishizaki I, Nukada T, Kamegaya E, Furuya S, Hirabayashi Y, Ikeda K, Hata H, Kobayashi H, Sora I, Yamamoto H: **Functional identification of ASCT1 neutral amino acid transporter as the predominant system for the uptake of L-serine in rat neurons in primary culture.** *Neurosci Res* 2004, **49**:101-11.
20. Broer A, Brookes N, Ganapathy V, Dimmer KS, Wagner CA, Lang F, Broer S: **The astroglial ASCT2 amino acid transporter as a mediator of glutamine efflux.** *J Neurochem* 1999, **73**:2184-94.
21. Weis S, Llenos IC, Dulay JR, Verma N, Sabunciyan S, Yolken RH: **Changes in region- and cell type-specific expression patterns of neutral amino acid transporter 1 (ASCT-1) in the anterior cingulate cortex and hippocampus in schizophrenia, bipolar disorder and major depression.** *J Neural Transm* 2007, **114**:261-71.
22. Borowsky B, Mezey E, Hoffman BJ: **Two glycine transporter variants with distinct localization in the CNS and peripheral tissues are encoded by a common gene.** *Neuron* 1993, **10**:851-63.
23. Lechner SM: **Glutamate-based therapeutic approaches: inhibitors of glycine transport.** *Curr Opin Pharmacol* 2006, **6**:75-81.
24. American Psychiatric Association: **DSM-IV: Diagnostic and Statistical Manual of Mental Disorders.** American Psychiatric Press, Washington, DC; 1994.
25. Lahiri DK, Nurnberger JI Jr: **A rapid non-enzymatic method for the preparation of HMW DNA from blood for RFLP studies.** *Nucleic Acids Res* 1991, **19**:5444.
26. **dbSNP database** [http://www.ncbi.nlm.nih.gov/SNP]
27. Reich DE, Cargill M, Bolk S, Ireland J, Sabeti PC, Richter DJ, Lavery T, Kouyoumjian R, Farhadian SF, Ward R, Lander ES: **Linkage disequilibrium in the human genome.** *Nature* 2001, **411**:199-204.
28. Shibata H, Joo A, Fujii Y, Tani A, Makino C, Hirata N, Kikuta R, Ninomiya H, Tashiro N, Fukumaki Y: **Association study of polymorphisms in the GluR5 kainate receptor gene (*GRIK1*) with schizophrenia.** *Psychiatr Genet* 2001, **11**:139-44.
29. Nickerson DA, Tobe VO, Taylor SL: **Polyphred: substitutions using fluorescence-based resequencing.** *Nucleic Acids Res* 1997, **25**:2745-2751.
30. Xie X, Ott J: **Testing linkage disequilibrium between a disease gene and marker loci.** *Am J Hum Genet* 1993, **53**:1107.
31. Abecasis GR, Cookson WO: **GOLD: graphical overview of linkage disequilibrium.** *Bioinformatics* 2000, **16**:182-183.
32. Sham P: **Statistics in human genetics.** Oxford University Press, New York; 1998.
33. Benjamini Y, Drai D, Elmer G, Kafkafi N, Golani I: **Controlling the false discovery rate in behavior genetics research.** *Behav Brain Res* 2001, **125**:279-284.
34. Ohashi J, Tokunaga K: **The power of genome-wide association studies of complex disease genes: statistical limitations of indirect approaches using SNP markers.** *J Hum Genet* 2001, **46**:478-482.
35. Coon H, Myles-Worsley M, Tiobech J, Hoff M, Rosenthal J, Bennett P, Reimherr F, Wender P, Dale P, Polloi A, Byerley W: **Evidence for a chromosome 2p13-14 schizophrenia susceptibility locus in families from Palau, Micronesia.** *Mol Psychiatry* 1998, **3**:521-527.
36. Straub RE, MacLean CJ, Ma Y, Webb BT, Myakishev MV, Harris-Kerr C, Wormley B, Sadek H, Kadambi B, O'Neill FA, Walsh D, Kendler KS: **Genome-wide scans of three independent sets of 90 Irish multiplex schizophrenia families and follow-up of selected regions in all families provides evidence for multiple susceptibility genes.** *Mol Psychiatry* 2002, **7**:542-559.
37. Bennett PJ, Hoff M, Rosenthal J, Zhao M, Coon H, Myles-Worsley M, Byerley WF: **Mutation screening of a neutral amino acid transporter, ASCT1, and its potential role in schizophrenia.** *Psychiatr Genet* 2000, **10**:79-82.
38. Skowronek MH, Georgi A, Jamra RA, Schumacher J, Becker T, Schmael C, Paul T, Deschner M, Hofels S, Wulff M, Schwarz M, Klopp N, Illig T, Propping P, Cichon S, Nothen MM, Schulze TG, Rietschel M: **No association between genetic variants at the ASCT1 gene and schizophrenia or bipolar disorder in a German sample.** *Psychiatr Genet* 2006, **16**:233-4.
39. Chen AC, Kalsi G, Brynjolfsson J, Sigmundsson T, Curtis D, Butler R, Read T, Murphy P, Barnard EA, Petursson H, Gurling HM: **Exclusion of linkage between schizophrenia and the gene encoding a neutral amino acid glutamate/aspartate transporter, *SLC1A5*.** *Am J Med Genet* 1997, **74**:50-2.
40. Tsai SJ, Cheng CY, Hong CJ, Liao DL, Hou SJ, Yen FC, Liou YJ: **Association study of polymorphisms in glycine transporter with schizophrenia.** *J Neural Transm* 2006, **113**:1545-9.
41. Jamra RA, Villela AW, Klein K, Becker T, Schulze TG, Schmael C, Deschner M, Klopp N, Illig T, Propping P, Cichon S, Rietschel M, Nothen MM, Schumacher J: **No association between genetic variants at the GLYT2 gene and bipolar affective disorder and schizophrenia.** *Psychiatr Genet* 2006, **16**:91.
42. **HapMap database** [http://www.hapmap.org]
43. Ponce J, Poyatos I, Aragon C, Gimenez C, Zafra F: **Characterization of the 5' region of the rat brain glycine transporter GLYT2 gene: identification of a novel isoform.** *Neurosci Lett* 1998, **242**:25-8.
44. Mills RE, Luttig CT, Larkins CE, Beauchamp A, Tsui C, Pittard WS, Devine SE: **An initial map of insertion and deletion (INDEL) variation in the human genome.** *Genome Res* 2006, **16**:1182-90.

Pre-publication history

The pre-publication history for this paper can be accessed here:

http://www.biomedcentral.com/1471-244X/8/58/prepub

Publish with **BioMed Central** and every scientist can read your work free of charge

"BioMed Central will be the most significant development for disseminating the results of biomedical research in our lifetime."

Sir Paul Nurse, Cancer Research UK

Your research papers will be:

- available free of charge to the entire biomedical community
- peer reviewed and published immediately upon acceptance
- cited in PubMed and archived on PubMed Central
- yours — you keep the copyright

Submit your manuscript here:
http://www.biomedcentral.com/info/publishing_adv.asp

J Hum Genet (2008) 53:863–866
DOI 10.1007/s10038-008-0318-7

SHORT COMMUNICATION

No association between the oligodendrocyte-related gene *PLP1* and schizophrenia in the Japanese population

Branko Aleksic · Masashi Ikeda · Ryoko Ishihara · Shinichi Saito · Toshiya Inada · Nakao Iwata · Norio Ozaki

Received: 3 April 2008 / Accepted: 21 June 2008 / Published online: 5 July 2008
© The Japan Society of Human Genetics and Springer 2008

Abstract *PLP1* is one of the major myelin-related genes. A large body of expression-based studies showed significantly lower levels of the *PLP1* messenger ribonucleic acid (mRNA) transcripts in schizophrenia. Moreover, one family-based study identified a weak association signal in a male subset using 487 Chinese family trios. We carried out a population-based association study between *PLP1* and schizophrenia in 1,640 subjects. Our data does not support genetic variation in close vicinity or within PLP1 locus as a susceptibility factor.

Keywords Genetic association · Myelination · X chromosome · Proteolipid protein 1

Introduction

Schizophrenia is a severe mental disorder with a global prevalence of 1%. It is known that a genetic component is in some degree responsible for its pathogenesis; however, specific factors have not yet been discovered. One hypothesis is that oligodendrocyte dysfunction may confer susceptibility for schizophrenia (Stewart and Davis 2004). This viewpoint is along the lines of a developmental model of etheogenesis, as environmental factors might interfere with cellular maturation of the central nervous system (CNS) (i.e., myelination). Moreover, certain alleles may exhibit protective (or inclining) properties toward impact of the deleterious environmental effect.

PLP1 (length 15.8 kbp, location Xq22) is one of the oligodendrocyte-related genes. Proteins encoded by the aforementioned gene (proteolipid protein 1 and its splicing variant DM20) are integral to membranes and together constitute almost 50% of the protein in CNS myelin. The primary role is thought to be related to the adhesion and stabilization of the extracellular myelin membrane surfaces (Klugmann et al. 1997). Mutations associated with *PLP1* are responsible for: (1) Pelizaeus-Merzbacher disease (OMIM #312080) characterized clinically by spastic quadriplegia, ataxia, and developmental delay (Johnson et al. 1991), and (2) spastic paraplegia type 2 (OMIM #312920).

A large number of expression- and animal-model-based studies have shown significantly lower levels of *PLP1* messenger ribonucleic acid (mRNA) in schizophrenia (Tkachev et al. 2003; Aston et al. 2004; Le-Niculescu et al. 2007; Sokolov 2007). One study examined the hypothesis of *PLP1* as candidate gene (Qin et al. 2005), and data provided evidence for genetic association with schizophrenia. As *PLP1* is indeed an attractive candidate gene for schizophrenia, we performed a gene-centric (Neale and Sham 2004) association study using a large Japanese sample to replicate previous findings.

B. Aleksic (✉) · R. Ishihara · S. Saito · N. Ozaki
Department of Psychiatry, Graduate School of Medicine, Nagoya University, Showa-ku, Tsurumai-Cho, 65, Nagoya, Aichi 466-8550, Japan
e-mail: branko@med.nagoya-u.ac.jp

B. Aleksic · M. Ikeda · N. Iwata
Department of Psychiatry, Graduate School of Medicine, Fujita Health University, Aichi, Japan

B. Aleksic · M. Ikeda · R. Ishihara · S. Saito · N. Iwata · N. Ozaki
CREST, Japan Science and Technology Agency, Saitama 332-0012, Japan

T. Inada
Seiwa Hospital, Institute of Neuropsychiatry, Tokyo, Japan

Table 1 Demographic data

Status	Gender	Number	Average age
Case	Male	418	40.27 ± 11.60
	Female	410	44.05 ± 15.10
Control	Male	410	40.78 ± 13.76
	Female	402	43.09 ± 13.91

Sample

Our sample consisted of 828 patients with schizophrenia (average age 42.14 ± 13.57 years) and 812 subjects with no personal or family history of psychiatric illness (average age 41.92 ± 13.87 years). The subjects consisted of Japanese individuals. Other relevant demographic data are shown in Table 1.

All schizophrenic patients met *Diagnostic and Statistical Manual of Mental Disorders, Fourth Edition* (DSM IV) criteria, reflected by consensual diagnosis of two experienced psychiatrists. Prior to inclusion in the control set, subjects were screened on the basis of a brief diagnostic interview. Subsequent to the study description, written informed consent was requested from each subject. This study was approved by the ethics committee at Nagoya University.

Methods

All association analyses were carried out by calculating the *P* values for each single nucleotide polymorphism (SNP) marker, and significance was determined at the 5% level using either the chi-square test or Fisher's exact test. All *P* values are two-sided. Gender was taken into account, with the male subjects contributing a single allele to the analysis and female subjects contributing two alleles. In other words, for an organism where the male is of heterogametic and the female is of homogametic gender, regarding the X-linked genes, males normally possess only one X chromosome. They are hemizygous for (nearly) all genes that are located on the X chromosome (such as *PLP1*). Therefore, the case cohort was stratified by gender and tested against corresponding gender-specific control groups. For the female cohort, association between genotypes and alleles of SNPs and schizophrenia susceptibility were measured. For the male analysis, only allele-wise association was measured.

Linkage disequilibrium (LD) block structure was assessed in accordance with the confidence intervals criteria (Gabriel et al. 2002). Log likelihood ratio tests for assessing haplotype-wise association between schizophrenia and combination of tagging SNPs was performed on the software UNPHASED v3.04 (Dudbridge 2003) with a permutation test for calculating empirical significance levels for differences in haplotype frequencies between case and control sets. Male samples (containing only one *PLP1* allele) allowed unambiguous assignments of haplotypes. For female subjects, haplotypes and their frequencies were assigned by an estimation maximization algorithm as implemented in UNPHASED v3.04. Due to inherent uncertainty of haplotype prediction for female subjects, these analyses were performed when the case cohort was stratified by gender and tested against corresponding gender-specific control groups.

Power was calculated in accordance with methods described by Skol et al. (2006). In brief, for predefined alpha level and model of inheritance, statistical power of any given sample is a function of sample and effect size. In other words, power is directly proportional to sample size on the one hand and minor allele frequency (MAF) and genotype relative risk (GRR) on the other hand.

To test for genetic association, the gene-based approach was implemented. This method implies inclusion of both gene and gene-adjacent regions in the association study (Neale and Sham 2004). Therefore, the screened region was extended 5 kb upstream of the annotated transcription start site and downstream at the end of the last *PLP1* exon. Later, by taking advantage of observed LD patterns in a predefined region, the most informative subset of SNPs was chosen from an open-access source. Specifically, polymorphisms were selected from the HapMap database (release #21; phase II; July 2006, population: Japanese in Tokyo) based on correlation coefficient (r^2) between two loci. In other words, if r^2 was 0.8 or greater, then only one of the two loci was selected for the association study. Moreover, SNPs with a minor allele population frequency less than 5% were excluded. Based on the aforementioned criteria, three SNPs (rs471416, rs2233696, and rs10521502) were selected for genotyping. The SNP from the initial report (Qin et al. 2005) that had been shown to be associated with schizophrenia (rs475827) failed to met tagging SNP criteria due to low minor allele frequency in the Japanese population (4.7%). However this SNP was included as well, as it is the SNP that was shown to be associated with schizophrenia. Moreover, analysis of HapMap data showed that allele frequencies of rs475827 in the Chinese population (MAF = 4.4%) were similar to those in the Japanese population (MAF = 4.7%). Analysis of LD pattern and distribution of common haplotypes for the SNPs (MAF > 5%) in the *PLP1* region (HapMap data) provided comparable results for both populations.

For SNPs on the X chromosome, assessment of deviation from Hardy-Weinberg equilibrium (HWE) was different. In detail, HWE can be tested using two null hypotheses: (1) allele frequencies between cases and controls are equal, and (2) HWE holds in females (Zheng et al. 2007).

Genomic deoxyribonucleic acid (DNA) was extracted from peripheral blood. Allelic discrimination assay (ABI Biosystem) was carried out for genotype characterization of the selected SNPs. For each 384-well plate, three non-template controls were included.

Results

Regarding tagging SNPs, a priori power analysis showed that our sample reached a level of 80% for detecting association under the multiplicative model of inheritance when GRR was set to 1.35 for gender-stratified data and MAF was 30% (which is the lowest MAF for tagging SNPs). In case of the SNP from the initial study that was shown to be associated with schizophrenia, our sample had 80% power for detecting association when GRR was set to 1.8 for gender-stratified data and MAF was set to 4%.

A priori evaluation of HapMap data regarding the *PLP1*-related region showed existence of one LD block spreading gene region. The same pattern was observed a posteriori by assessing our experimental data. Analyses were carried out using Haploview v3.32 (Barrett et al. 2005). Deviation from HWE was not detected. Statistical analysis did not provide sufficient evidence for the genetic association (Tables 2, 3, 4).

Discussion

The common disease–common variant hypothesis states that multigenetic diseases, such as schizophrenia, that are evolutionarily neutral (i.e., a little or no effect on reproductive fitness) during human history may be significantly influenced by common variants (Lander 1996). Therefore, if an allelic variant at the disease susceptibility locus is responsible for predisposition to a common complex disease, then allele-, genotype-, or haplotype-wise association tests will detect such variants (or a tagging SNP that is in LD with a deleterious allele).

The first and only indication that the *PLP1*-related region harbors variation that might influence susceptibility to schizophrenia was provided by Qin and colleagues, who identified a weak association signal between rs475827 (intronic SNP, MAF = 4.4%) and schizophrenia in a male cohort only using 487 Chinese family trios. However, our study, based on adequate statistical power, did not support association between schizophrenia and the *PLP1* locus. As lack of replication may also be falsely negative, possibly due to the lack of adequately powered sample sizes, we performed a power analysis of the sample from the initial

Table 3 Results (*P* values single marker)

dbSNP	Males	Females	
	Allele	Allele	Genotype
rs475827	0.439	0.205	0.314
rs471416	0.943	0.959	0.573
rs2233696	0.276	0.381	0.626
rs10521502	0.596	0.559	0.634

Table 4 Results (*P* value multimarker analysis, sliding window)

dbSNP	Males			Females		
	2 SNPs	3 SNPs	4 SNPs	2 SNPs	3 SNPs	4 SNPs
rs475827	0.685	0.682	0.881	0.929	0.817	0.796
rs471416	0.585			0.877		
rs2233696		0.844			0.751	
rs10521502	0.747			0.826		

SNP single nucleotide polymorphism

Table 2 Genotype distribution

dbSNP	Status	Allele ($p > q$)	Position	Males		Females				
				Allele		Genotype			Allele	
				p/-	*q/-*	*p/p*	*p/q*	*q/q*	*p*	*q*
rs475827	Case	A > G	Upstream	25	393	2	60	348	64	756
	Control			19	391	2	45	355	49	755
rs471416	Case	C > T	Upstream	254	164	158	181	71	497	323
	Control			251	159	147	192	63	486	318
rs2233696	Case	A > G	Intronic	141	277	55	183	172	293	527
	Control			154	256	58	189	155	305	499
rs10521502	Case	C > T	Downstream	130	288	46	167	197	259	561
	Control			120	290	44	177	181	265	539

report (Qin et al. 2005). This analysis showed that the sample used in our research had a higher level of power. In other words, regarding our sample, to detect association when minor allele frequency was set to population-based data (rs475827), in order to achieve 80% of power, GRR has to be more than 1.8 (for gender-stratified data). On the other hand, regarding the schizophrenia-associated SNP rs475827, sample ($N_{\text{male trios}} = 253$) in the original report (Qin et al. 2005), the same minor allele frequency and GRR had only 51% of power for detecting association.

In light of the fact that a large body of expression-based studies have shown significantly lower levels of *PLP1* mRNA transcripts in animal models of schizophrenia or in brain tissue from deceased schizophrenic patients in comparison with healthy controls (Tkachev et al. 2003; Aston et al. 2004; Le-Niculescu et al. 2007), our results suggest that the downregulation is not influenced by the predisposing common genetic variants within or in close vicinity to the *PLP1* gene region.

An emerging body of work over the last 5 years has implicated myelin/glia related dysfunction in schizophrenia (Tkachev et al. 2003; Aston et al. 2004; Le-Niculescu et al. 2007). Moreover, downregulation of at least some of the many abnormally expressed myelin-associated genes in schizophrenia might be influenced by a systematic mechanism such as methylation, as in case of *SOX10* (Iwamoto et al. 2005), or a transcriptional factor that can exert an effect on multiple myelin-associated genes such as *QKI* (Aberg et al. 2006; Haroutunian et al. 2006) rather than an SNP in the regulatory region of each abnormally expressed myelin-associated genes. Interestingly, Aberg and colleagues showed that human *QKI* mRNA levels can account for a high proportion of normal interindividual mRNA expression variation (and covariation) of *PLP1*. Therefore, it is not surprising that we could not replicate the finding from the original report.

The chances are that new experimental methodologies (such as oligonucleotide arrays) will eventually increase signal-to-noise ratio and give us a better perspective regarding genetic factors influencing complex disorders and probably higher reproducibility of the results from genetic association studies.

Acknowledgments This work was supported in part by research grants from the Ministry of Education, Culture, Sports, Science and Technology of Japan, the Ministry of Health of Japan, Labor and Welfare, Grant-in-Aid for Scientific Research on Pathomechanisms of Brain Disorders from the Ministry of Education, Culture, Sports, Science and Technology of Japan, MEXT Academic Frontier, the Japan Health Sciences Foundation (Research on Health Sciences focusing on Drug Innovation), and the Core Research for Evolutional Science and Technology.

Conflict of interest statement None declared.

References

Aberg K, Saetre P, Jareborg N, Jazin E (2006) Human QKI, a potential regulator of mRNA expression of human oligodendrocyte-related genes involved in schizophrenia. Proc Natl Acad Sci USA 103:7482–7487

Aston C, Jiang L, Sokolov BP (2004) Microarray analysis of postmortem temporal cortex from patients with schizophrenia. J Neurosci Res 77:858–866

Barrett JC, Fry B, Maller J, Daly MJ (2005) Haploview: analysis and visualization of LD and haplotype maps. Bioinformatics 21:263–265

Dudbridge F (2003) Pedigree disequilibrium tests for multilocus haplotypes. Genet Epidemiol 25:115–121

Gabriel SB, Schaffner SF, Nguyen H, Moore JM, Roy J, Blumenstiel B, Higgins J, DeFelice M, Lochner A, Faggart M, Liu-Cordero SN, Rotimi C, Adeyemo A, Cooper R, Ward R, Lander ES, Daly MJ, Altshuler D (2002) The structure of haplotype blocks in the human genome. Science 296:2225–2229

Haroutunian V, Katsel P, Dracheva S, Davis KL (2006) The human homolog of the QKI gene affected in the severe dysmyelination "quaking" mouse phenotype: downregulated in multiple brain regions in schizophrenia. Am J Psychiatry 163:1834–1837

Iwamoto K, Bundo M, Yamada K, Takao H, Iwayama-Shigeno Y, Yoshikawa T, Kato T (2005) DNA methylation status of SOX10 correlates with its downregulation and oligodendrocyte dysfunction in schizophrenia. J Neurosci 25:5376–5381

Johnson VP, Carpenter NJ, Kelts KA (1991) Pelizaeus-Merzbacher disease: clinical and DNA-linkage study of an extended family. Am J Med Genet 41:355–361

Klugmann M, Schwab MH, Puhlhofer A, Schneider A, Zimmermann F, Griffiths IR, Nave KA (1997) Assembly of CNS myelin in the absence of proteolipid protein. Neuron 18:59–70

Lander ES (1996) The new genomics: global views of biology. Science 274:536–539

Le-Niculescu H, Balaraman Y, Patel S, Tan J, Sidhu K, Jerome RE, Edenberg HJ, Kuczenski R, Geyer MA, Nurnberger JI Jr, Faraone SV, Tsuang MT, Niculescu AB (2007) Towards understanding the schizophrenia code: an expanded convergent functional genomics approach. Am J Med Genet B Neuropsychiatr Genet 144:129–158

Neale BM, Sham PC (2004) The future of association studies: gene-based analysis and replication. Am J Hum Genet 75:353–362

Qin W, Gao J, Xing Q, Yang J, Qian X, Li X, Guo Z, Chen H, Wang L, Huang X, Gu N, Feng G, He L (2005) A family-based association study of PLP1 and schizophrenia. Neurosci Lett 375:207–210

Skol AD, Scott LJ, Abecasis GR, Boehnke M (2006) Joint analysis is more efficient than replication-based analysis for two-stage genome-wide association studies. Nat Genet 38:209–213

Sokolov BP (2007) Oligodendroglial abnormalities in schizophrenia, mood disorders and substance abuse. Comorbidity, shared traits, or molecular phenocopies? Int J Neuropsychopharmacol 10:547–555

Stewart DG, Davis KL (2004) Possible contributions of myelin and oligodendrocyte dysfunction to schizophrenia. Int Rev Neurobiol 59:381–424

Tkachev D, Mimmack ML, Ryan MM, Wayland M, Freeman T, Jones PB, Starkey M, Webster MJ, Yolken RH, Bahn S (2003) Oligodendrocyte dysfunction in schizophrenia and bipolar disorder. Lancet 362:798–805

Zheng G, Joo J, Zhang C, Geller NL (2007) Testing association for markers on the X chromosome. Genet Epidemiol 31:834–843