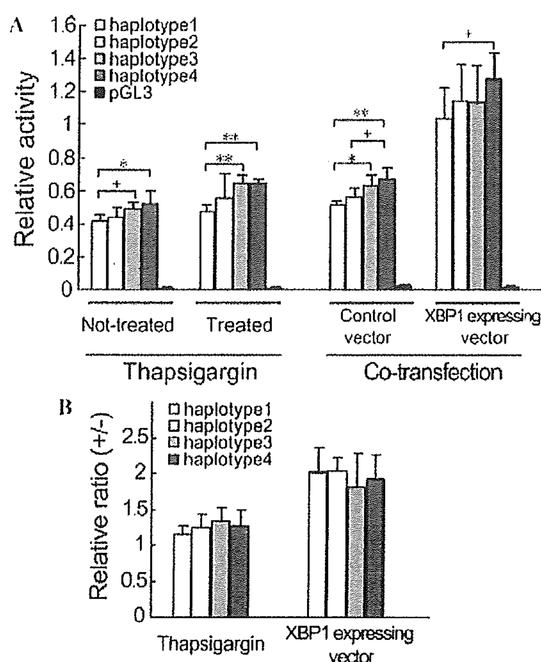

Fig. 3. Effects of haplotype for promotor activity. (A) Basal activity and the activity treated with 300 nM thapsigargin of the construct having haplotype 3 or haplotype 4 were significantly higher than that of haplotype 1, or show a tendency higher than that of haplotype 2. Y-axis is relative activity of firefly luciferase of reporter plasmid to *Renilla* luciferase of reference plasmid. Values are means ± SD. Asterisk indicates $p < 0.05$, double asterisk, $p < 0.01$ and plus sign $0.05 < p < 0.1$ (Two-sample t test). Assay was performed independently four times. (B) The response to ER stress or XBP1 co-transfection. There was no difference among four haplotypes in the response rate. Vertical axis indicates the relative ratio of the activity treated with thapsigargin to the activity not treated, or the ratio of the activity in the cells co-transfected with spliced form XBP1-expressing vector to that in the cells with control vector. Values are means ± SD.

structs. SHSY5Y cells were transfected by the constructs with pRL-CMV vectors and the relative activity of firefly luciferase (pGL3) to *Renilla* luciferase (pRL) was examined. Among four constructs, the activity of constructs having haplotype 3 and haplotype 4, which are the risk for Japanese bipolar samples and NIMH trios, were significantly higher than that of haplotype 1 (Fig. 3A). Similar difference was observed during the stimulation by 300 nM thapsigargin, the ER Ca^{2+}-ATPase inhibitor. The response to ER stress did not differ between haplotypes. Co-transfection with spliced form XBP1-expressing vector enhanced the promotor activity. This enhancement was observed similarly in these four constructs (Fig. 3B).

Genotyping of the genomic DNA and mRNA in the brain samples

The result of genetic analysis in NIMH trios is compatible with a hypothesis that the haplotype 3 confers a risk only when it is paternally transmitted. A possible explanation for such a phenomenon, that is parent-of-origin effect, is genomic imprinting [2]. To test whether or not HSPA5 shows monoallelic expression in the brain, we genotyped the SNP7 at 3′-UTR in genomic DNA and cDNA obtained from postmortem brain tissues [prefrontal or frontal cortex of bipolar disorder ($n = 41$), schizophrenia ($n = 46$), depression ($n = 12$), and control ($n = 41$)]. The 81 samples having heterozygous genotype in genomic DNA also showed heterozygous genotype in cDNA, which did not suggest monoallelic expression.

Discussion

In this study, we found that there are four haplotypes in the promotor region of HSPA5. A promotor assay revealed that these polymorphisms affect the promotor activity. We also demonstrated that the haplotype of *HSPA5* (*GRP78/BiP*) gene was nominally associated with bipolar disorder in Japanese population. However, because (1) the global p value was not significant, (2) the association was not replicated in an independent sample set, and (3) no association was found in NIMH trio samples, this could be a type I error. Since the global p value was significant in the samples with family history of mood disorder, it would be interesting to test the association in an independent sample set of bipolar disorder with family history. Because parent-of-origin effect is suggested in bipolar disorder [2,32–34] although controversial [35–38], we hypothesized that parent-of-origin effect might confound the findings in NIMH trios. Thus, we performed the hypothesis-generating analysis, which showed significant over-transmission of haplotype 3 from paternal side. This finding made us to hypothesize that HSPA5 is subjected to genomic imprinting. However, monoallelic expression was not observed in the frontal cortex. This did not support our hypothesis. Because imprinting is dependent on the region of the brain [39,40], a possibility that HSPA5 is imprinted in the specific brain region cannot be totally excluded. However, before considering such possibility, this finding of nominal association in paternal transmission should be tested in an independent sample set.

The haplotype in the upstream region showing the nominal association in Japanese and NIMH altered the promotor activity. In this experimental condition, these haplotypes were associated with higher promotor activity. HSPA5 is known to have three ER stress response elements (ERSE, consensus sequence; CCAAT-N9-CCACG) (ERSE1–3) in the promotor region [41]. All the SNPs (SNP1–4) located not within but upstream of ERSE, and thus are unlikely to alter ER stress response. Indeed, there was no difference of response rate among haplotypes against ER stress induced by thapsigargin, while the risk haplotype showed higher activity both in the basal level and after induction of ER stress. In addition to ERSE, *HSPA5* expression is controlled by binding sites of many transcription factors such as ATF4 and AP-1 [42,43]. However, the three SNPs in the risk haplotype do not alter the binding sites of known transcription factors. Unknown transcription factor may determine the basal promotor

activity. Higher promotor activity of the risk is seemingly inconsistent with the reported evidence that valproate improves the ER stress response, and *HSPA5* response to ER stress was impaired in bipolar disorder [4]. This may be due to the cell-type difference or because we used artificial vectors having only promotor regions. It is also possible that higher basal promotor activity might paradoxically result in impaired ER stress response. Although precise mechanism is still unclear, altered regulation of *HSPA5* may contribute to the pathophysiology of bipolar disorder.

The role of ER stress response in the brain is little clarified. Recently, ER stress response was reported to be critical for trafficking of AMPA-type glutamate receptors [44,45]. GluR1 accumulated in the ER of mutant *Caenorhabditis elegans* lacking XBP1 or IRE1. On the other hand, the role of lithium and valproate in AMPA GluR1 receptor trafficking was reported [46]. Altered ER stress response system may contribute to the pathophysiology of bipolar disorder via altered trafficking of AMPA receptors.

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Genomewide High-Density SNP Linkage Analysis of 236 Japanese Families Supports the Existence of Schizophrenia Susceptibility Loci on Chromosomes 1p, 14q, and 20p

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The Japanese Schizophrenia Sib-Pair Linkage Group (JSSLG) is a multisite collaborative study group that was organized to create a national resource for affected sib pair (ASP) studies of schizophrenia in Japan. We used a high-density single-nucleotide–polymorphism (SNP) genotyping assay, the Illumina BeadArray linkage mapping panel (version 4) comprising 5,861 SNPs, to perform a genomewide linkage analysis of JSSLG samples comprising 236 Japanese families with 268 nonindependent ASPs with schizophrenia. All subjects were Japanese. Among these families, 122 families comprised the same subjects analyzed with short tandem repeat markers. All the probands and their siblings, with the exception of seven siblings with schizoaffective disorder, had schizophrenia. After excluding SNPs with high linkage disequilibrium, we found significant evidence of linkage of schizophrenia to chromosome 1p21.2-1p13.2 (LOD = 3.39) and suggestive evidence of linkage to 14q11.2 (LOD = 2.87), 14q11.2-q13.2 (LOD = 2.33), and 20p12.1-p11.2 (LOD = 2.33). Although linkage to these regions has received little attention, these regions are included in or partially overlap the 10 regions reported by Lewis et al. that passed the two aggregate criteria of a meta-analysis. Results of the present study—which, to our knowledge, is the first genomewide analysis of schizophrenia in ASPs of a single Asian ethnicity that is comparable to the analyses done of ASPs of European descent—indicate the existence of schizophrenia susceptibility loci that are common to different ethnic groups but that likely have different ethnicity-specific effects.

Introduction

Schizophrenia (MIM 181500) is a common disorder, with a lifetime morbidity risk of 1%. A large number of family, twin, and adoption studies have revealed that individual differences in susceptibility are predominantly genetic, with a heritability of 0.70–0.85 and a 10-fold increased risk in siblings of probands (Levinson and Mowry 2000). More than 20 genome scans for susceptibility loci for schizophrenia have been completed, and evidence satisfying genomewide significance levels for linkage to schizophrenia was obtained for chromosome regions 6p24-p22 (MIM 600511) (Straub et al. 1995), 1q21-q22 (MIM 604906) (Brzustowicz et al. 2000), 13q32-q34 (MIM 603176) (Blouin et al. 1998), 10p14 (DeLisi et al. 2002*b*), and 10q25.3-q26.3 (Williams et al. 2003). Linkage for other regions—including 8p22-p21 (MIM 603013) (Kendler et al. 1996; Blouin et al. 1998), 6q21-q25 (MIM 603175) (Cao et al. 1997; Lindholm et al. 2001), 22q11-q12 (MIM 600850) (Pulver et al. 1994; Schizophrenia Linkage Collaborative Group

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for Chromosomes 3 and 8 1996), and 5q21-q33 (MIM 181510) (Bassett et al. 1988; Paunio et al. 2001)—has been reported multiple times. However, none of the above-named regions has been identified consistently in a majority of the genome scans. It is possible that loci with small populationwide effects hinder confirmation of linkage because replication of linkage data requires a larger sample population than the original data set (Suarez et al. 1994) and because the samples for most genome scans of schizophrenia have been small, typically 20–100 families.

Some problems of power and replication can be addressed by meta-analysis. Lewis and colleagues (2003) used the rank-based genome-scan meta-analysis (GSMA) method to analyze 20 complete genome scans for susceptibility loci for schizophrenia. In GSMA, the autosomes were divided into 30-cM bins, and the evidence of linkage in each study was rank ordered across bins with and without weights for sample size. The average ranks across studies were evaluated for statistically significant evidence of linkage in several ways. Lewis et al. (2003) concluded that schizophrenia loci are highly likely to be present in one or more of the following regions: 1p13.3-q23.3, 2p12-q23.3, 3p25.3-p22.1, 5q23.2-q34, 6pter-p21.1, 8p22-p21.1, 11q22.3-q24.1, 14pter-q13.1, 20p12.3-p11, and 22pter-q12.3, all of which met two aggregate criteria for linkage. Another meta-analysis found significant results only for chromosomes 8p, 13q, and 22q (Badner and Gershon 2002). However, meta-analysis has limitations (Levinson et al. 2003). One is that meta-analysis methods may not distinguish several weakly linked loci in the same region. This issue can be addressed by pooling the raw genotyping data for meta-analysis. Analysis of a multicenter sample of 779 pedigrees did not yield significant evidence of linkage of 22q to schizophrenia (Mowry et al. 2004); however, those authors suggested that collaborative pooling of data sets was limited by intersite differences in sampling frames, population ethnicity, and genotyping methods.

The largest genomewide linkage findings to date were reported by DeLisi and colleagues (2002*b*), who studied 294 families with 382 nonindependent affected sib pairs (ASPs) with schizophrenia or schizoaffective disorder from the United States, the United Kingdom, Italy, Chile, and Belgium. Williams and colleagues (2003) described linkage findings in 272 families with 353 nonindependent ASPs from the United Kingdom, Sweden, and the United States. Among these ASPs, 287 nonindependent ASPs in 231 families received a diagnosis of schizophrenia or schizoaffective disorder. Straub and colleagues (2002) described linkage findings in 270 families with 261 nonindependent ASPs with schizophrenia or poor-outcome schizoaffective disorder from Ireland and Northern Ireland. The Irish families were ethnically homogeneous, and most of the pedigrees in the other two studies were of European origin. The narrow ethnic distributions of these sample populations could have influenced the results, because an ethnically diverse study population has increased potential for variation, which could result in heterogeneity at certain susceptibility loci. A recent study found ethnic heterogeneity between European and East Asian populations in allelic association of the 102T/C polymorphism of the *HTR2A* gene (MIM 182135) with schizophrenia (Abdolmaleky et al. 2004). This type of heterogeneity compounds the recognized difficulty in characterizing genetically complex diseases for which the magnitude of the effect of any one locus is unknown.

The Japanese Schizophrenia Sib-Pair Linkage Group (JSSLG), a multisite collaborative study group, was established in 1997 as a national resource for genetic studies of schizophrenia. An initial genomewide linkage study was performed with 417 STR markers in 130 families; however, no loci with significant linkage to schizophrenia were detected (JSSLG 2003). We recruited additional families to participate in the JSSLG study and analyzed 236 families with 268 nonindependent ASPs with a high-density SNP linkage mapping set. High-density SNP linkage mapping systems provide significantly improved levels of information extraction with extremely high accuracy, particularly when founder genotypes are unavailable (Sawcer et al. 2004).

Material and Methods

Subjects

Linkage of genetic loci to schizophrenia was analyzed in Japanese families with at least two available siblings who had received the diagnosis of schizophrenia or schizoaffective disorder. A total of 236 families with 602 individuals were recruited at 24 centers across Japan (table 1). Of these, 122 families with 315 individuals comprised the same subjects analyzed by STR markers that we reported elsewhere (JSSLG 2003). Each family member received the diagnosis on the basis of the DSM-IV structured clinical interview. Each face-to-face interview was conducted by two experienced interviewers. In addition to direct interviews, all available medical records and information from relatives and hospital staff were considered. Inclusion criteria for this collaborative sample recruitment were DSM-IV-defined schizophrenia for probands and schizophrenia or schizoaffective disorder for affected siblings. Seven siblings with schizoaffective disorder were included. All participants and their parents were of Japanese descent. The study protocol was approved by the ethics committee of each institution, and written informed consent was obtained from all subjects.

Table 1

JSSLG Subjects

		No. of JSSLG Families					
		Both Parents[b]		One Parent[b]		No Parent[b]	
Center[a]	No. of Families	2 Affected Sibs	3 Affected Sibs	2 Affected Sibs	3 Affected Sibs	2 Affected Sibs	3 Affected Sibs
Hakkaido University	2	0	0	0	0	1	1
Hirosaki University	1	0	0	0	0	0	1
Minami Hanamaki National Hospital	18	5	0	8	0	5	0
Tohoku University	1	0	0	0	0	1	0
Fukushima Medical University	4	0	0	2	0	2	0
Niigata University	19	3	0	2	0	14	0
University of Tsukuba	16	0	0	3	2	10	1
Teikyo University Ichihara Hospital	23	0	0	2	0	21	0
RIKEN Brain Science Institute	7	5	0	2	0	0	0
Juntendo University	3	2	0	1	0	0	0
Toho University	10	0	0	0	0	10	0
Tokyo Institute of Psychiatry	1	0	0	0	0	0	1
Nihon University	11	1	1	0	0	9	0
Teikyo University	4	0	0	2	0	2	0
National Center of Neurology and Psychiatry	5	0	0	0	0	5	0
Fujita Health University	7	0	0	0	0	7	0
Osaka Medical College	4	0	0	0	0	4	0
Okayama University	33	0	0	2	0	27	4
University of Occupational and Environmental Health	10	1	0	0	0	9	0
Kyushu University	2	0	0	0	0	2	0
Kurume University	7	0	0	0	0	7	0
Saga Medical School	22	5	1	5	1	10	0
Nagasaki University	19	7	0	7	1	3	1
Kagoshima University	7	0	1	1	0	5	0
Total	236	29	3	37	4	154	9

[a] In order of location from north to south.
[b] Available for genotyping.

Genotyping

The Illumina SNP-based Linkage Panel IV was used for genotyping. The panel includes 5,861 SNP markers distributed evenly across the genome. The average and median intervals between markers were 503 kb (0.64 cM) and 301 kb (0.35 cM), respectively. The largest interval between successfully genotyped markers was 4.9 Mb (8.8 cM) on chromosome Xp21. The Illumina markers were typed with the Illumina BeadStation 500G, in accordance with the manufacturer's standard recommendations.

Statistical Analysis

Multipoint linkage analysis was performed along the entire length of each chromosome with the MERLIN program (Center of Statistical Genetics) developed by Abecasis et al. (2002). Both the nonparametric linkage (NPL) Z score and nonparametric LOD score, calculated with the Kong and Cox (1997) linear model, were extracted from the MERLIN runs and were used to generate graphic plots of the whole-genome scan results. Because linkage disequilibrium (LD) between closely spaced SNP markers can falsely inflate linkage statistics, we used the SNPLINK program (Webb et al. 2005; Institute of Cancer Research), which removes LD from the marker sets in an automated fashion. Because the program considers LD between pairs of adjacent SNPs, the possibility of high LD between nonadjacent SNPs but low LD between adjacent SNPs, such as a situation in which there was high LD between SNPs 41 and 43 and low LD between SNPs 41 and 42 and between SNPs 42 and 43, was examined with the Haploview program. Because no empirical justification to remove LD by any criteria has been published, we tested the significant and suggestive regions, using a range of criteria from $r^2 = 0.4$, and gradually decreased the thresholds to $r^2 = 0.05$. The linkage panel includes 28 SNPs from the pseudoautosomal regions of the X chromosome (20 from the short arm; 8 from the long arm). Because no currently available multipoint linkage program can integrate data from X-linked and pseudoautosomal markers in a single analysis, each pseudoautosomal region was analyzed separately, as though it were an independent autosomal chromosome. The results of these analyses were then combined with those from the standard X-linked markers. Empirical P values were calculated for the NPL Z and LOD scores via simulation. MERLIN was used to

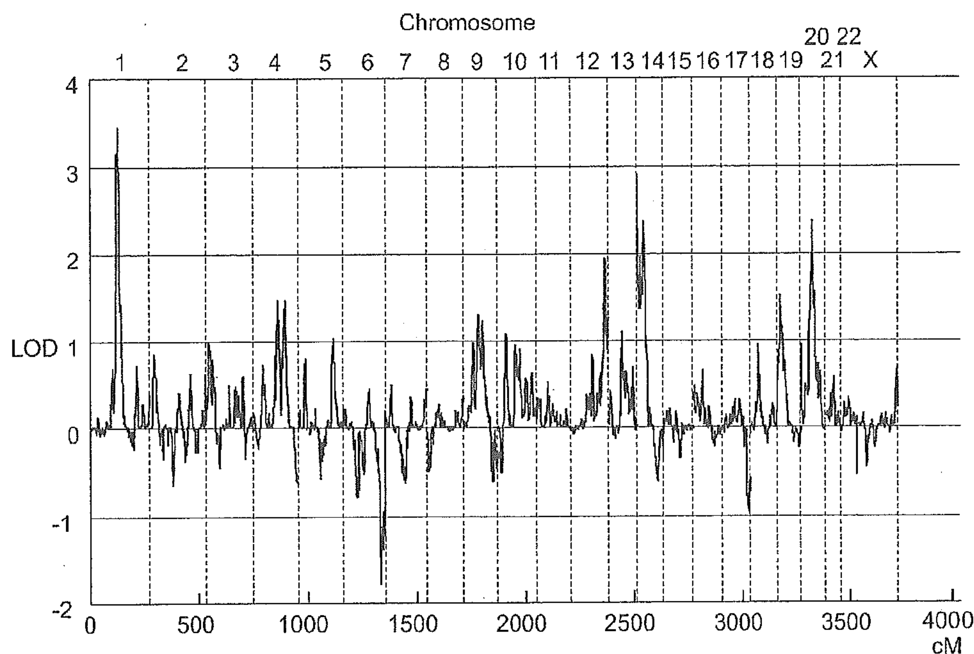

Figure 1 Multipoint nonparametric LOD score (Kong and Cox 1997) of genomewide scan for JSSLG ASPs with schizophrenia

generate 50,000 replicates of families identical to those in our sample. Markers with similar allele frequencies were also generated under the assumption of no linkage. Linkage analyses were then performed on these unlinked replicates, and peaks of NPL *Z* and LOD scores separated by at least 30 cM on each chromosome were recorded for each simulation. Simulation studies of our genome scan suggested that, on average, an NPL *Z* of 2.87 and a LOD of 2.06 per genome scan would have been expected, whereas an NPL *Z* of 3.48 and a LOD of 3.07 would have been expected to occur only once in every 20 genome scans in the absence of linkage. Therefore, these values correspond to "suggestive" and "significant" thresholds for genomewide significance, as defined by Lander and Kruglyak (1995). The GeneFinder program (Liang et al. 2001; Chiu et al. 2002; Glidden et al. 2003) was used to obtain 95% CIs for the locations of linked loci. The information content of the genotypes was estimated by MERLIN, with use of entropy information described by Kruglyak et al. (1996). Simulations suggested that our study had a power of >0.99, 0.79, 0.38, and 0.05 to detect a susceptibility locus of λs = 3, 2, 1.5, and 1.25 for schizophrenia, with a genomewide significance of $P = .05$.

Results

Among our Japanese family members, we observed an average minor-allele frequency of 0.29 and a mean heterozygosity of 0.37. These values were identical to those in Asian populations in the Illumina Linkage IV Panel. In our Japanese population, 125 SNPs were not polymorphic. The call rate (percentage of successful genotype calls among subjects) was used as a measure of quality. The average call rate was 98.5%, and we excluded 10 SNPs with call rates of <90%. The rate of Mendelian inconsistency or impossible recombination identified by the MERLIN program was 0.027% in the families with parents available for genotyping. Because the low heterozygosity of SNPs means that only 37% of genotyping errors will appear as Mendelian inconsistencies (Abecasis et al. 2002), the approximate genotyping error rate was estimated to be 0.073%.

Results of the linkage analysis are presented in figure 1. One region, 1p21.1, showed genomewide significance ($P < .05$) on the basis of simulation studies (LOD = 3.39; NPL *Z* = 3.96) with a 95% CI of 102.0–111.9 Mb (National Center for Biotechnology Information [NCBI] build 35). We also obtained suggestive evidence of linkage to chromosome 14q11.2 (LOD = 2.87; NPL *Z* = 3.14), with a 95% CI of 19.4–34.9 Mb; chromosome 14q12 (LOD = 2.33; NPL *Z* = 2.95), with a 95% CI of 19.4–34.9 Mb; and chromosome 20p11.2 (LOD = 2.33; NPL *Z* = 3.10), with a 95% CI of 16.0–33.2 Mb (table 2). Notable results were also obtained for chromosomes 4q24 (LOD = 1.44; NPL *Z* = 2.32), 4q31.3 (LOD = 1.44; NPL *Z* =

Table 2

Chromosome Regions with Genomewide Significant and Suggestive Linkage to Schizophrenia in 268 Nonindependent JSSLG ASPs

Peak SNP	Chromosome Region	Distance from pter Marker (cM)	Position[a] (Mb)	NPL Z (*P*)	LOD[b] (*P*)	95% CI SNP Region[a]	95% CI Position[a] (Mb)	95% CI Chromosome Region
rs2048839	1p21.1	126.18	105.7	3.96 (.00004)	3.39 (.00004)	*rs1445225–rs575208*	102.0–111.9	1p21.2-p13.2
rs1319956	14q11.2	.00	19.4	3.14 (.0009)	2.87 (.0001)	*rs1319956–rs8904*	19.4–34.9	14q11.2-q13.2
rs7149108	14q12	31.14	32.0	2.95 (.002)	2.33 (.0005)	*rs1319956–rs8904*	19.4–34.9	14q11.2-q13.2
rs7988	20p11.2	53.08	23.3	3.10 (.001)	2.33 (.0005)	*rs775133–rs663550*	16.0–33.2	20p12.1-q11.2

[a] NCBI build 35.

[b] Calculated with the Kong and Cox (1997) linear model.

2.42), 12q24.3 (LOD = 1.91; NPL Z = 2.67), and 19p13.3 (LOD = 1.49; NPL Z = 2.32).

Among 5,736 SNPs, 22 pairs of nonadjacent SNPs were in LD with $r^2 > 0.05$ but no adjacent SNPs were in LD with $r^2 < 0.05$. However, no such pairs were located in the significant and suggestive regions. The LOD scores were not changed by decreasing the thresholds to remove LD in the SNPLINK program, because pairs of adjacent SNPs showed high LD ($r^2 > 0.4$) or no or very low LD ($r^2 < 0.01$) in these regions.

Discussion

In our previous study of 130 families (JSSLG 2003), we did not observe any significant or suggestive evidence of linkage with schizophrenia. Of the 236 families examined in the present study, 122 had been analyzed previously. The present study revealed significant and suggestive evidence of linkage of specific chromosome regions to schizophrenia. The larger number of families and increased information extracted by the high-density SNP linkage system used in the present study may have contributed to the present results. The overall genetic linkage information content per 3-cM interval increased from 0.48 in our previous study (JSSLG 2003) to 0.72 in the present study. In addition to the increase in extractable information, high-throughput DNA typing technology is advantageous because it is accurate, fast, and requires little DNA. The genotyping error rate was ~0.073% in the present study. Although error rates are rarely published—and when they are expressed, the terminology varies greatly—it is noteworthy that microsatellite error rates of 0.1%–12.7% per reaction have been reported (Brzustowicz et al. 1993; Ginot et al. 1996; Ghosh et al. 1997; Ewen et al. 2000; Sobel et al. 2002; Weeks et al. 2002). Abecasis et al. (2001) reported that error rates of just 1% can reduce observed LOD scores by as much as 50%.

Our strongest finding was significant evidence of linkage of schizophrenia to the region 1p21-p13. To our knowledge, studies of linkage to schizophrenia have not focused on linkage to this region. However, this region overlaps a telomeric part of bin 1.6, which showed evidence of linkage to schizophrenia in the meta-analysis reported by Lewis et al. (2003) (table 2). A small peak LOD score for this region was observed in a cohort in the Central Valley of Costa Rica (DeLisi et al. 2002*a*). An NPL score of 2.72 for region 1p21 was observed in seven families with schizophrenia or schizophrenia spectrum personality disorders (Pulver et al. 2000). The *NTNG1* gene (MIM 608818) is located on 1p13.3 and may be a candidate gene for schizophrenia susceptibility. Association between specific haplotypes encompassing alternatively spliced exons of *NTNG1* and schizophrenia was observed in a Japanese population (Aoki-Suzuki et al. 2005).

Suggestive evidence of linkage to 14q11.2-q13.2 was also obtained in the present study. One region with NPL scores >2.0 in Arab-Israeli families was 14q11.1-q11.2 (Lerer et al. 2003). Potential linkage of schizophrenia to 14q13 was reported for the Maryland epidemiologic sample comprising 44 families of European descent (NPL = 2.57; *P* = .005) (Blouin et al. 1998). A mother and daughter who received the diagnoses of schizophrenia and schizophrenia comorbid with mild learning disability, respectively, possessed a balanced reciprocal translocation t(9,14)(q34.2;q13), and the *NPAS3* gene (MIM 609430) on 14q13.1 (32.5–33.3 Mb) was disrupted (Kamnasaran et al. 2003; Pickard et al. 2005). The region of 14q11.2-q13.2 is included in bin 14.1, which showed evidence of linkage in the meta-analysis reported by Lewis et al. (2003) (table 2).

Suggestive evidence of linkage to 20p11.2 was also obtained in the present study. Linkage of 20p11 with bipolar disorders has been reported (Radhakrishna et al. 2001; McInnis et al. 2003). This region is included in bin 20.2, which showed evidence of linkage with schizophrenia in the meta-analysis reported by Lewis et al. (2003) (table 2).

In the present study, all of the regions that showed significant and suggestive evidence of linkage to schizophrenia are included in or partially overlap the 10 regions that passed the two aggregate criteria of a meta-analysis (Lewis et al. 2003), although these regions have

not received much attention (Owen et al. 2004). Therefore, the presence of susceptibility loci for schizophrenia in both European and Asian populations in these regions is plausible, although these loci may have larger populationwide effects on schizophrenia in Asian populations than in European populations. Additional larger studies of Asian populations might validate the hypothesis (Hwu et al. 2005). In conclusion, the present JSSLG linkage study of Japanese families—which is one of the largest genomewide ASP analyses of a single ethnicity for schizophrenia to date and is comparable to genomewide ASP analyses of families of European descent with schizophrenia—supports the existence of schizophrenia susceptibility loci common to different ethnic groups but with possible ethnic-specific effects.

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Web Resources

The URLs for data presented herein are as follows:

Center of Statistical Genetics, http://csg.sph.umich.edu/ (for the MERLIN program)
GeneFinder, http://www.biostat.jhsph.edu/~wmchen/gf.html
Haploview, http://www.broad.mit.edu/mpg/haploview/
Institute of Cancer Research, http://www.icr.ac.uk/cancgen/molgen/MolPopGen_Bioinformatics.htm (for the SNPLINK program)
NCBI, http://www.ncbi.nlm.nih.gov/mapview/map_search.cgi?taxid=9606 (for map view build 35 and identification of candidate genes in locus of interest)
Online Mendelian Inheritance in Man (OMIM), http://www.ncbi.nlm.nih.gov/Omim/ (for schizophrenia, 6p24-p22, 1q21-q22, 13q32-q34, 8p22-p21, 6q21-q25, 22q11-q12, 5q21-q33, *HTR2A*, *NTNG1*, and *NPAS3*)

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Association Between Polymorphisms in the Promoter Region of the Sialyltransferase 8B (*SIAT8B*) Gene and Schizophrenia

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Background: *Sialyltransferase 8B (SIAT8B) and 8D (SIAT8D) are two polysialyltransferases that catalyze the transfer of polysialic acid (PSA) to the neural cell adhesion molecule 1 (NCAM1). PSA modification of NCAM1 plays an important role in neurodevelopment of the brain and disruption of this process is postulated as an etiologic factor in psychiatric disorders. Altered levels of the PSA-NCAM1 in the brain of schizophrenics have been reported, suggesting a role for this molecule in the disorder.*

Methods: *We performed an association study of single nucleotide polymorphisms (SNPs) within SIAT8B and SIAT8D, using 188 schizophrenics and 156 age and gender matched controls. All genotypes were determined by polymerase chain reaction (PCR) amplification and direct sequencing.*

Results: *Two polymorphisms, −1126T> C and −851T> C, located in the promoter region of SIAT8B showed nominally significant association with schizophrenia (allelic associations,* p = *.014 and* p = *.007, respectively), and haplotypes constructed from three additional SNPs located in the same linkage disequilibrium block were associated with schizophrenia. Furthermore an in vitro promoter assay revealed that a reporter construct containing a risk haplotype for SIAT8B had significantly higher transcriptional activity compared with one containing a protective haplotype (*p = *.021). In contrast, no significant association was observed between any variations in SIAT8D and schizophrenia.*

Conclusions: *The present study suggests that functional promoter SNPs of SIAT8B could confer a risk for schizophrenia in the Japanese population.*

Key Words: SIAT8D, polysialyltransferase, neural cell adhesion molecule, oligosaccharide, hippocampus, luciferase assay

Schizophrenia is a common and complex mental disorder that is clinically characterized by delusions, conceptual disorganization, hallucinatory behavior, cognitive dysfunction, blunted affect and psychosocial impairment. The worldwide prevalence of the disorder is approximately 1%, regardless of race, gender and economic conditions. The etiologic bases remain unknown, however, from twin, family and adoption studies, heritable factors make a strong contribution (~50%) to the development of schizophrenia (Gottesman 1991).

The neural cell adhesion molecule 1 (NCAM1) is known to regulate neuronal processes such as neuronal migration (Hu et al 1996; Ono et al 1994), axon pathfinding (Tang et al 1992), neurite outgrowth (Angata et al 1997; Nakayama et al 1995), spatial learning and memory (Cremer et al 1994; Eckhardt et al 2000) and synaptogenesis (Seki and Rutishauser 1998). We previously reported an association between NCAM1 and bipolar disorder (Arai et al 2004). The functions of NCAM1 are fine tuned by the addition of polysialic acid (PSA), which is dynamically involved in cell-cell and cell-matrix recognition (Bruses and Rutishauser 2001; Kiss and Muller 2001). PSA ligated-NCAM1 (PSA-NCAM1) is widely expressed in the embryonic and neonatal brain, but expression is highly restricted in the adult central nervous system, to areas such as the hippocampus and olfactory bulb, that maintain a permanent capacity for neurogenesis (Seki and Arai 1993; Kiss et al 2001). PSA-NCAM1 formation is continuously generated by two polysialyltranferases, sialyltransferase 8B (SIAT8B or sialyltransferase X, STX) and 8D (SIAT8D or polysialyltransferase, PST), in the developing and adult brain (Nakayama et al 1998).

Several lines of evidence support the hypothesis that dysregulation of PSA-NCAM1 in the brain increases the vulnerability to schizophrenia. First, Barbeau et al (1995) reported a reduction in anti-PSA immunoreactive cells in the hippocampi of schizophrenic brains, leading to a possible dysfunction of synaptic connectivity in this region. Secondly, reduced olfactory bulb volumes have been reported in first episode patients with schizophrenia (Turetsky et al 2003). This structural disturbance could be partly attributable to the dysfunction of PSA-NCAM1 during neurodevelopment, since a similar phenotype was observed in *Ncam1*-deficit mice (Tomasiewicz et al 1993). Thirdly, *Siat8b*-deficient mice exhibited reduced PSA in the dentate gyrus of the hippocampus (Angata et al 2004). Disturbances of the anatomical organization and hippocampal function have long been implicated in the etiology of schizophrenia (Harrison 2004). Additionally mice lacking *Siat8d* showed disturbed cognitive performances (Eckhardt et al 2000), a common defect of schizophrenia (Mohamed et al 1999). Fourthly, *SIAT8B* maps to chromosome 15q26, a region reported in a genome scan of Eastern Quebec families as a common susceptibility region for both schizophrenia and bipolar disorder (Maziade et al 2005). The chromosomal region of *SIAT8D* at 5q21 is also reported as a susceptibility locus for schizophrenia in an analysis of reconstructed ancestral haplotypes (Lindolm et al 2004).

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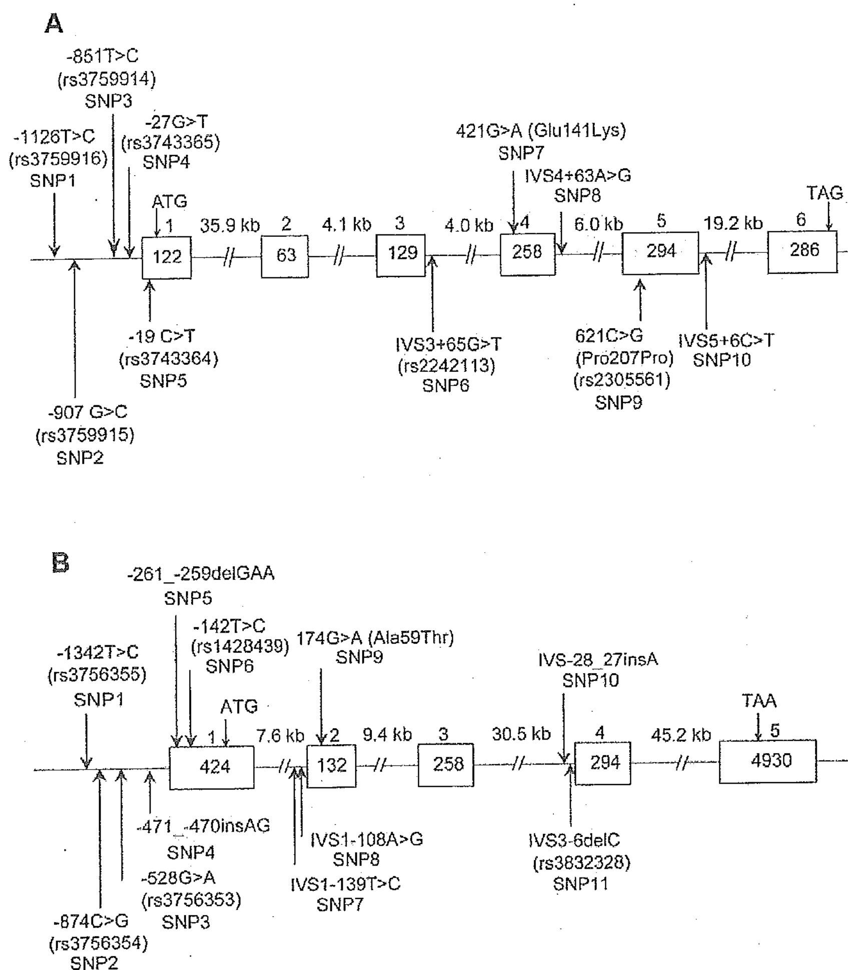

Figure 1. Genomic structures and locations of polymorphic sites for human sialyltransferases. **(A)** Sialyltransferase 8B (*SIAT8B*) and **(B)** Sialyltransferase 8D (*SIAT8D*). Exons are numbered and the location of the initiation codon (ATG) and stop codon (TAA or TAG) are denoted. The sizes of exons (base) and introns (kilobase, kb) are provided. The rs number is the National Center for Biotechnology Information single nucleotide polymorphism (SNP) cluster identification number from the dbSNP database (http://www.ncbi.nih.gov/SNP/).

Therefore, in this study we performed genetic analysis of *SIAT8B* and *SIAT8D* as compelling candidates for schizophrenia.

Methods and Materials

Subjects

For the case-control study, we recruited 188 unrelated schizophrenics, of whom 98 were male (mean age 54.2 ± 7.9) and 90 female (mean age 54.8 ± 12.7). All patients were diagnosed according to the Diagnostic and Statistical Manual of Mental Disorders, Fourth Edition (DSM-IV) (American Psychiatric Association 1994), to give best-estimate lifetime diagnosis, with consensus from at least two experienced psychiatrists. All available medical records and family informant reports were also taken into consideration. Mentally healthy control subjects were recruited from among hospital staff and company employees, whose relatives within the third degree were also free from psychoses according to the interview of blood donors. They comprised 82 males (mean age 55.9 ± 17.3) and 74 females (mean age 53.9 ± 12.4). All participants in this study were derived from western part of Japan.

The present study was approved by the Ethics Committees of Tokyo Institute of Psychiatry, RIKEN and Okayama University, and all participants provided their informed consent.

Determination of Genomic Structures

The genomic structures of *SIAT8B* and *SIAT8D* were based on the University of California, Santa Cruz (UCSC) May 2004 draft assembly of the human genome (http://genome.ucsc.edu/). All exons conformed to sequences RefGene NM_006011 (*SIAT8B*) and NM_005668 (*SIAT8D*), with the 'A' of the ATG initiation codon counted as +1. The 'rs' numbers from the dbSNP database (http://www.ncbi.nlm.nih.gov/projects/SNP/) are shown where known (Figure 1).

Screening and Genotyping of Polymorphisms

Genomic DNA was isolated from whole blood according to standard procedures. All exons, splice boundaries and 5′ upstream regions of *SIAT8B* and *SIAT8D* were screened for polymorphisms by direct sequencing of PCR products, from 20 unrelated schizophrenia samples. The PCR fragments were amplified using sets of primers designed manually (Table 1) and one of the following DNA polymerases, according to the template, TaKaRa Taq (Takara Bio Inc., Shiga, Japan), Go-Taq DNA polymerase (Promega, Madison, Wisconsin), Expand Long Template PCR System (Roche Diagnotics GmbH, Mannheim, Germany) or Advantage-GC Genomic PCR kit (BD Biosciences, Clontech, Palo Alto, California). Detailed information on amplification conditions is available upon request. Direct sequencing

Table 1. PCR Primers Used in the Detection of Nucleotide Variants in *SIAT8B* and *SIAT8D*

Region	Primers (F, forward; R, reverse)	Product Size (bp)	3′ End of Primer
SIAT8B			
5′ upstream	(F) 5′-aggacattccatgtagcttctccaa-3′	551	1298 bp upstream to A of ATG initiation codon
	(R) 5′-ccaggtgctgatctttatctccattt-3′		797 bp upstream to A of ATG initiation codon
5′ upstream/Exon 1	(F) 5′-ttattgatcttagggtcattgagagtcc-3′	1082	984 bp upstream to A of ATG initiation codon
	(R) 5′-ccgatttcttcttcgatctctga-3′		75 downstream to A of ATG initiation codon
Exon 2	(F) 5′-caagtgagaacctggctacttact-3′	546	237 bp upstream to exon 2
	(R) 5′-acccatctgcattttccatctctg-3′		200 bp downstream to exon 2
Exon 3	(F) 5′-ttatcaccctccttcctgtctcca-3′	534	206 bp upstream to exon 3
	(R) 5′-cgctaggcttgtctcgggttttta-3′		153 bp downstream to exon 3
Exon 4	(F) 5′-gccaccaccgtagttgcttctg-3′	680	181 bp upstream to exon 4
	(R) 5′-ctctcttcccctctcctccattgat-3′		196 bp downstream to exon 4
Exon 5	(F) 5′-acagggtctagcaaagttctaggta-3′	729	203 bp upstream to exon 5
	(R) 5′-caaaattggcatgccagatgaaac-3′		185 bp downstream to exon 5
Exon 6	(F) 5′-ctgtacccacaacaggtttgttttg-3′	773	228 bp upstream to exon 6
	(R) 5′-acaccttcttcctgtggcctatct-3′		212 bp downstream to G of TAG stop codon
SIAT8D			
5′ upstream	(F) 5′-ttcgctgtgctgcaaagaggaaatt-3′	614	1771 bp upstream to A of ATG initiation codon
	(R) 5′-aagagcaaagtcctctctaaaagtc-3′		1206 bp upstream to A of ATG initiation codon
	(F) 5′-agaggtactcagagtgaacaatcga-3′	600	1305 bp upstream to A of ATG initiation codon
	(R) 5′-ctaaacacacaccctcacacgcaaa-3′		754 bp upstream to A of ATG initiation codon
	(F) 5′-tgcactttccagattgagtaat-3′	573	825 bp upstream to A of ATG initiation codon
	(R) 5′-acaccttgtgcattggaggtg-3′		294 bp upstream to A of ATG initiation codon
5′ upstream/Exon 1	(F) 5′-acgttactctccaagcagcct-3′	743	394 bp upstream to A of ATG initiation codon
	(R) 5′-ccacaaccacacagagcaagtt-3′		195 bp downstream to exon 1
Exon 2	(F) 5′-cgtgtagattcttgtcctgatagtc-3′	613	173 bp upstream to exon 2
	(R) 5′-gtgaagtatgttaaatatcagtactctgc-3′		256 bp downstream to exon 2
Exon 3	(F) 5′-gtatacagtcattaagaatctgtgcaaatga-3′	712	201 bp upstream to exon 3
	(R) 5′-gaatcttgttgctatgacaggatcac-3′		198 bp downstream to exon 3
Exon 4	(F) 5′-ggatattcttagacaacaggttgaattc-3′	792	234 bp upstream to exon 4
	(R) 5′-tgaacacacataaataagtgccaggt-3′		212 bp downstream to exon 4
Exon 5	(F) 5′-ggtgagattctagagtaatattcacctac-3′	760	240 bp upstream to exon 5
	(R) 5′-ttactaggacccttaaagtcaaa-3′		187 bp downstream to A of TAA stop codon

PCR, polymerase chain reaction; SIAT, sialyltransferase.

of the PCR products was performed using the BigDye Terminator Cycle Sequencing FS Ready Reaction kit (Applied Biosystems, Foster City, California) and ABI PRISM 3100 Genetic Analyzer (Applied Biosystems).

Statistical Analysis

For genotype distribution of each polymorphism, deviation from Hardy-Weinberg equilibrium was examined by chi-square test. To evaluate the significance of any statistical differences observed, Fisher's exact probability test or the Monte-Carlo method implemented in the CLUMP program (T1 mode, number of simulations set to 10,000) (Sham and Curtis 1995) were used when appropriate. Calculations of linkage disequilibrium (LD) between variants and the estimation of haplotype frequencies were performed using the Arlequin 2.0 software (http://lgb.unige.ch/arlequin/). Power calculations were performed using the Power Calculator (http://calculators.stat.ucla.edu/powercalc/).

In Vitro Promoter Assay

The fragments spanning each of the $-1126\text{T} > \text{C}$ and $-851\text{T} > \text{C}$ sites of *SIAT8B* were amplified from DNA samples using the forward primer, 5′-gtacgctatgacggtaccAGGACATTCCATGTAGCTTCTCCAA [3′ end at nt −1298; the underlined sequence was added to introduce a Kpn I site (ggtacc)] and the reverse primer, 5′-atcgtagctagcggtaccCCGATTTCTTCTTCGATCTCTGA (3′ end at nt +76; the underlined sequence was added to introduce a Kpn I site) by the Advantage-GC Genomic PCR kit (BD Biosciences, Clontech). In order to examine the promoter activities of putative risk and protective haplotypes separately, DNA samples from homozygous individuals for each haplotype were amplified. The amplified (1420 bp genomic + Kpn I site-containing flanking) fragments were cloned into the pGL3-basic plasmid lacking enhancer sequences (Promega). The transfections were done according to the manufacturer's recommendations (Invitrogen, Carlsbad, California). In brief, human neuroblastoma IMR-32 cells were plated at 10^5 cells per well in a 24 well plate and grown in Dulbecco's Minimal Essential Medium (Sigma, St. Louis, Missouri) supplemented with 10% fetal bovine serum. *SIAT8B* expression in the human neuroblastoma IMR-32 cell line has been confirmed by real-time RT-PCR (Brocco et al 2003). One μg of the pGL3-basic plasmid with a cloned promoter fragment was transiently co-transfected into the cells with .1 μg of the pKL-TK plasmid (an internal standard reporter) (Promega) using LipofectAMINE 2000 (Invitrogen). The growth medium was replaced after 6 hours. After an additional 48 hour incubation period, transfected cells were washed with phosphate-buffered saline and incubated with shaking in cell lysis buffer for 15 min at room temperature. Thereafter, the luciferase assay was performed using the PicaGene Dual SeaPansy kit according to the protocol of manufacturer (Toyo Ink, Tokyo, Japan). The activity was measured by a TopCount detector (PerkinElmer, Boston, MA).

Luciferase activity was not detectable when we transfected the *SIAT8B* promoter construct into COS-1 cells that are deemed

Table 2. Genotype and Alletic Distributions of the *SIAT8B* and *SIAT8D* Polymorphisms

Polymorphisms	*n*	Genotype Counts (frequency)			*p* value[a]	Allele Counts (frequency)		*p* value[a]
SIAT8B								
−1126T > C (SNP1)		T/T	T/C	C/C		T	C	
Schizophrenia	188	86 (.46)	79 (.42)	23 (.12)	.046	251 (.67)	125 (.33)	.014
Control	156	56 (.36)	67 (.43)	33 (.21)		179 (.57)	133 (.43)	
−907G > C (SNP2)		G/G	G/C	C/C		G	C	
Schizophrenia	188	44 (.23)	97 (.52)	47 (.25)	.256	185 (.49)	191 (.51)	.220
Control	156	49 (.31)	71 (.46)	36 (.23)		169 (.54)	143 (.46)	
−851T > C (SNP3)		T/T	T/C	C/C		T	C	
Schizophrenia	188	88 (.47)	77 (.41)	23 (.12)	.030	253 (.67)	123 (.33)	.007
Control	156	55 (.35)	68 (.44)	33 (.21)		178 (.57)	134 (.43)	
−27G > T (SNP4)		G/G	G/T	T/T		G	T	
Schizophrenia	188	65 (.35)	98 (.52)	25 (.13)	.391	228 (.61)	148 (.39)	.478
Control	156	64 (.41)	70 (.45)	22 (.14)		198 (.63)	114 (.37)	
−19C > T (SNP5)		C/C	C/T	T/T		C	T	
Schizophrenia	188	65 (.35)	98 (.52)	25 (.13)	.391	228 (.61)	148 (.39)	.478
Control	156	64 (.41)	70 (.45)	22 (.14)		198 (.63)	114 (.37)	
IVS3+65 G > T (SNP6)		G/G	G/T	T/T		G	T	
Schizophrenia	188	79 (.42)	92 (.49)	17 (.09)	.049	250 (.66)	126 (.34)	.367
Control	156	81 (.52)	56 (.36)	19 (.12)		218 (.70)	94 (.30)	
421G > A (Glu > Lys) (SNP7)		G/G	G/A	A/A		G	A	
Schizophrenia	188	187 (.99)	1 (.01)	0 (.00)	1.000	375 (1.00)	1 (.00)	1.000
Control	156	156 (1.00)	0 (.00)	0 (.00)		312 (1.00)	0 (.00)	
IVS4+63 A > G (SNP8)		A/A	A/G	G/G		A	G	
Schizophrenia	188	185 (.98)	3 (.02)	0 (.00)	1.000	373 (.99)	3 (.01)	1.000
Control	156	154 (.99)	2 (.01)	0 (.00)		310 (.99)	2 (.01)	
621C > G (Pro > Pro) (SNP9)		C/C	C/G	G/G		C	G	
Schizophrenia	188	171 (.91)	17 (.09)	0 (.00)	.700	359 (.95)	17 (.05)	.707
Control	156	144 (.92)	12 (.08)	0 (.00)		300 (.96)	12 (.04)	
IVS5+6 C > T (SNP10)		C/C	C/T	T/T		C	T	
Schizophrenia	188	171 (.91)	17 (.09)	0 (.00)	.700	359 (.95)	17 (.05)	.707
Control	156	144 (.92)	12 (.08)	0 (.00)		300 (.96)	12 (.04)	
SIAT8D								
−1342T > C (SNP1)		T/T	T/C	C/C		T	C	
Schizophrenia	188	103 (.55)	69 (.37)	16 (.09)	.363	275 (.73)	101 (.27)	.177
Control	156	77 (.49)	59 (.38)	20 (.13)		213 (.68)	99 (.32)	
−874C > G (SNP2)		C/C	C/G	G/G		C	G	
Schizophrenia	188	103 (.55)	68 (.36)	17 (.09)	.244	274 (.73)	102 (.27)	.080
Control	156	73 (.47)	62 (.40)	21 (.13)		208 (.67)	104 (.33)	
−528G > A (SNP3)		G/G	G/A	A/A		G	A	
Schizophrenia	188	99 (.53)	72 (.38)	17 (.09)	.644	270 (.72)	106 (.28)	.450
Control	156	78 (.50)	59 (.38)	19 (.12)		215 (.69)	97 (.31)	
−471_−470insAG (SNP4)		D/D	D/I	I/I		D	I	
Schizophrenia	188	187 (.99)	1 (.01)	0 (.00)	1.000	375 (1.00)	1 (.00)	1.000
Control	156	155 (.99)	1 (.01)	0 (.00)		311 (1.00)	1 (.00)	
−261_−259delGAA (SNP5)		I/I	I/D	D/D		I	D	
Schizophrenia	188	128 (.68)	52 (.28)	8 (.04)	.685	308 (.82)	68 (.18)	.438
Control	156	99 (.63)	50 (.32)	7 (.04)		248 (.79)	64 (.21)	
−142T > C (SNP6)		T/T	T/C	C/C		T	C	
Schizophrenia	188	103 (.55)	67 (.36)	18 (.10)	.270	273 (.73)	103 (.27)	.095
Control	156	73 (.47)	62 (.40)	21 (.13)		208 (.67)	104 (.33)	
IVS1−139T > C (SNP7)		T/T	T/C	C/C		T	C	
Schizophrenia	188	177 (.94)	10 (.05)	1 (.01)	1.000	364 (.97)	12 (.03)	1.000
Control	156	147 (.94)	9 (.06)	0 (.00)		303 (.97)	9 (.03)	
IVS1−108A > G (SNP8)		A/A	A/G	G/G		A	G	
Schizophrenia	188	183 (.97)	5 (.03)	0 (.00)	.733	371 (.99)	5 (.01)	.734
Control	156	153 (.98)	3 (.02)	0 (.00)		309 (.99)	3 (.01)	
174G > A (Ala59Thr) (SNP9)		G/G	G/A	A/A		G	A	
Schizophrenia	188	187 (.99)	1 (.01)	0 (.00)	.592	375 (1.00)	1 (.00)	.593
Control	156	154 (.99)	2 (.01)	0 (.00)		310 (.99)	2 (.01)	
IVS3−28_−27insA (SNP10)		D/D	D/I	I/I		D	I	
Schizophrenia	188	187 (.99)	0 (.00)	1 (.01)	.702	374 (.99)	2 (.01)	1.000
Control	156	155 (.99)	1 (.01)	0 (.00)		311 (1.00)	1 (.00)	

Table 2. (continued)

Polymorphisms	n	Genotype Counts (frequency)			p value[a]	Allele Counts (frequency)		p value[a]
IVS3−6delC (SNP11)		I/I	I/D	D/D		I	D	
Schizophrenia	188	187 (.99)	1 (.01)	0 (.00)	1.000	375 (1.00)	1 (.00)	1.000
Control	156	156 (1.00)	0 (.00)	0 (.00)		312 (1.00)	0 (.00)	

D, deletion; I, insertion; SNP, single nucleotide polymorphism; SIAT, sialyltransferase.
[a]Differences in genotypic and allelic distributions were evaluated by Fisher's exact test.

to lack endogenous *SIAT8B* and *SIAT8D* expressions (Nakayama et al 1995, 1998).

Results

Detection of the *SIAT8B* and *SIAT8D* Gene Polymorphisms

The genomic layout of the human *SIAT8B* and *SIAT8D* genes, which span 70.3 kb on 15q26 and 96.1 kb on 5q21, respectively, are shown in Figure 1. The transcription start sites of both genes have been confirmed experimentally (Scheidegger et al 1995; http://dbtss.hgc.jp/). The *SIAT8B* gene is composed from 6 exons, and *SIAT8D* of 5 exons. Based on this structural information, we performed mutation screening of the genes, inspecting all exons, splice boundaries and the 5′ upstream region using 20 randomly chosen schizophrenic patients. We identified 10 polymorphisms including three novel variants [421G > A (Glu141Lys), IVS4+63A > G and IVS5+6C > T] in *SIAT8B*, and 11 polymorphisms in *SIAT8D*, six in the 5′ upstream region, four within introns and a 174G > A (Ala59Thr) substitution in exon 2. Six polymorphisms in *SIAT8D* were novel (those denoted without rs numbers in Figure 1).

Genetic and Transcriptional Analyses of *SIAT8B*

Genotypic distributions of all the polymorphisms showed no deviations from Hardy-Weinberg equilibrium (Table 2). Transmission patterns are unknown in complex genetic diseases including psychiatric disorders. In general, genotypic analysis is more powerful than allelic analysis to detect association when a risk allele has a recessive effect, and allelic analysis is more advantageous when a risk allele has a dominant effect. The frequency of minor alleles for three variants, IVS4+63A > G (SNP8), 621C > G (Pro207Pro) (SNP9) and IVS5+6C > T (SNP10) were 5% or less, and the novel variant, 421A (Lys141, SNP7) was detected in just one schizophrenic patient. Of the 10 polymorphisms, −1126T > C (SNP1) and −851T > C (SNP3) showed nominally significant allelic associations with schizophrenia (−1126T > C: $p = .014$, OR = 1.49, 95% CI = 1.09 – 2.03; −851T > C: $p = .007$, OR = 1.55, 95% CI = 1.13 – 2.11) (Table 2). We observed marginal genotypic associations between schizophrenia and each of the SNPs, −1126T > C (SNP1) ($p = .05$), −851T > C (SNP3) ($p = .03$) and IVS3+65G > T (SNP6) ($p = .05$). We conducted power calculations based on an arbitrary assumption of relative risk and frequency of risk allele. When a relative risk of 2.0 was assumed, samples in this study displayed a power of .926 to detect a significant association ($\alpha < .05$, frequency of risk allele = .3). With a relative risk of 1.5, the power was estimated at .552 to detect significant association ($\alpha < .05$, frequency of risk allele = .3).

Using six common variants (minor allele frequencies > 5%), we examined LD structures between them (Table 3). The schizophrenia associated SNP1 and SNP3 were in strong LD. SNPs 1 to 5 (−19C > T) were in substantial LD with each other ($D' > .9$ and $r^2 > .3$; D', normalized D; r^2, squared correlation coefficient) and considered to be in the same LD block, while there was an LD gap between SNP5 and SNP6. Next, we performed haplotype analyses in a sliding window fashion on the LD block spanning SNP1 to SNP5. All of the two-SNP-based haplotypes except for SNP4-SNP5 showed significant association with schizophrenia (the most significant window was SNP1-2 and its global p value was .0034). The three-, four-, and five-marker haplotypes also showed significant global association with disease (the most significant window was SNP1-2-3, giving global $p = .0026$). The individual haplotype frequencies of the five-SNP-based haplotypes in control and schizophrenia groups are shown in Table 4. The T (SNP1)-G (SNP2)-T (SNP3)-G (SNP4)-C (SNP5) haplotype was more frequent in schizophrenia than in controls ($p = .026$), while the C-G-C-G-C was less frequent in the disease group compared to controls ($p = .013$), suggesting that the former may be a risk haplotype and the latter a protective one.

Since the haplotype block constructed by SNPs1 to 5 spans the transcription regulatory region of *SIAT8B*, we examined the promoter activity of the putative risk and protective haplotypes in an in vitro luciferase reporter system. The DNA fragment with the T-G-T-G-C risk haplotype linked to the luciferase gene directed 1.84-fold greater transcriptional activity than that with the protective C-G-C-G-C haplotype ($p = .021$) (Figure 2).

Genetic Analysis of *SIAT8D*

The case-control analysis of *SIAT8D* showed no evidence of association with schizophrenia, for neither allelic nor genotypic distributions of the 11 SNPs (Table 2). As in the case of *SIAT8B*,

Table 3. Pairwise Linkage Disequilibrium Between Polymorphisms of *SIAT8B*

Polymorphisms	−1126T > C (SNP1)	−907G > C (SNP2)	−851T > C (SNP3)	−27G > T (SNP4)	−19C > T (SNP5)	IVS3+65G > T (SNP6)
−1126T > C (SNP1)		1.000 (.942)	.974 (1.000)	.951 (1.000)	.951 (1.000)	.068 (.494)
−907G > C (SNP2)	.630 (.453)		1.000 (.961)	.949 (.984)	.949 (.984)	.324 (.165)
−851T > C (SNP3)	.950 (.976)	.630 (.460)		.952 (1.000)	.951 (1.000)	.068 (.484)
−27G > T (SNP4)	.393 (.319)	.620 (.605)	.407 (.312)		1.000 (1.000)	.337 (.173)
−19C > T (SNP5)	.393 (.319)	.620 (.605)	.393 (.312)	1.000 (1.000)		.337 (.173)
IVS3+65G > T (SNP6)	.001 (.062)	.038 (.014)	.002 (.059)	.028 (.010)	.028 (.010)	

Values above the diagonal shows standardized D' in control (schizophrenia) groups, and values below the diagonal shows r^2 (squared correlation coefficient) in controls (schizophrenia). SNP, single nucleotide polymorphism; SIAT, sialyltransferase.

Table 4. Estimated Individual Haplotype Frequencies of *SIAT8B*

Haplotype					Frequency[a]		
−1126T > G (SNP1)	−907G > C (SNP2)	−851T > C (SNP3)	−27G > T (SNP4)	−19C > T (SNP5)	Control (n = 156)	Schizophrenia (n = 188)	Individual p Value[b]
C	G	C	G	C	.416	.321	.013
T	C	T	G	C	.104	.110	.804
T	C	T	T	T	.355	.387	.428
T	G	T	G	C	.105	.167	.026

Haplotypes with frequencies greater than .01 are shown.
SIAT, Sialyltransferase; SNP, single nucleotide polymorphism.
[a]Haplotype frequencies were estimated by the Arlequin software.
[b]Calculated by Fisher's exact test.

the informative nucleotide variants (minor allele frequencies > 5%) were clustered in the 5′ upstream region of *SIAT8D*, and these included −1342T > C (SNP1), −874C > G (SNP2), −528G > A (SNP3), −261_−259delGAA (SNP5) and −142T > C (SNP6) (Table 2). We examined the pair-wise LD measure between the 'common' SNPs, and found that SNP1, SNP2, SNP3, SNP5 and SNP6 were in one LD block (for all SNP pairs, D' = .908 − 1.00 and r^2 = .436 − 1.00). Sliding window haplotype analyses of this LD block detected no significant associations between *SIAT8D* and schizophrenia (data not shown).

Discussion

While both *SIAT8B* and *SIAT8D* regulate the annexation of negatively charged oligosaccharides onto PSA, this study suggests that only *SIAT8B* may play a role in conferring susceptibility to Japanese schizophrenia. Despite the fact that polysaccharides are abundantly expressed in the brain and their crucial role in development and synaptic plasticity in the central nervous system, there have been no investigations of association between polysaccharide-synthesizing genes and schizophrenia. To our knowledge this is the first molecular genetic report suggesting an involvement of polysaccharide dysregulation in the pathophysiology of this disease.

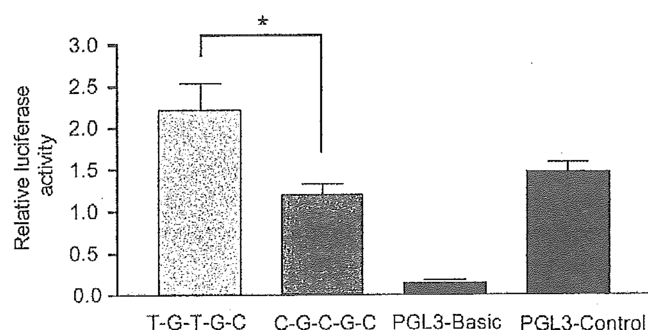

Figure 2. Effect of the *SIAT8B* haplotypes on promoter activity. Each value represents the mean ± SEM for five independent transfection experiments performed in duplicate. The 5′-regulatory haplotype fragments (1420 bp) of *SIAT8B* were constructed from (−1126T > C)-(−907G > C)-(−851T > C)-(−27G > T)-(−19C > T). The vector pGL3-Basic plasmid with cloned polymerase chain reaction (PCR) fragment was transiently co-transfected into IMR-32 cells with the pRL-TK plasmid (an internal standard reporter) using LipofectAMINE2000. The vector pGL3-Control containing the SV40 promoter was used as a positive control, and the pGL3-Basic lacking enhancer sequences was used as a negative control. * $p < .05$ by unpaired, t-test with two-tailed.

The schizophrenia associated haplotype block of *SIAT8B* resided in the putative promoter of transcriptional binding sites using the databases (TFSEARCH, http://mbs.cbrc.jp/research/db/TFSEARCHJ.html; MACH™, http://www.gene-regulation.com/cgi-bin/pub/programs/match/bin/match.cgi) demonstrated several putative consensus motifs including those for CCAAT, MZF1, CREB, GATA, TATA and SP1 (Figure 3). The disease associated −1126T > C (SNP1) and −851T > C (SNP3) encompass the CCAAT box and GATA-1/-2 binding sites, respectively. The T-to-C transition at nt −851 destroys the GATA-1/-2 binding consensus, and this is consistent with the transfection data showing that the −851T construct evoked a greater transcriptional activity than the −851C construct. In the mouse, *Siat8b* gene expression is driven by Sp1 binding motifs located in a TATA-less GC-rich domain (Yoshida et al 1996), and by the cAMP-CREB cascade, which potentiates the survival and proliferation of adult hippocampal neurons (Nakagawa et al 2002).

The allele frequencies of disease-associated SNP1 and 3 in the JSNP database (http://snp.ims.u-tokyo.ac.jp/) are closer to those in the patient group than those in the controls of this study. The precise reason for this is unknown. One of the possibilities may be that our controls and their relatives were free from the history of psychiatric illnesses, while the origins of samples used in the databases are unknown.

The risk-conferring promoter haplotype of *SIAT8B* detected in this study showed an increased transcriptional capability, which might result in the promotion of PSA transfer. Seemingly in contrast, histological examinations using postmortem brains (Barbeau et al 1995) and gene knockout experiments in mice (Angata et al 2004) suggested PSA reduction as a schizophrenia-related trait. However, Brocco et al (2003) reported that over-expression of *Siat8b* fusion protein in primary rat hippocampal cell cultured induced abnormal accumulation of *Siat8b* on the surface of neuronal cells leading to neuronal death. Therefore, it is likely that *SIAT8B* mRNA levels are strictly controlled for normal neuronal development and maintenance of proper neuronal function, since either an over-production or insufficiency of PSA could disturb integrity of this system. Disturbances in the fine-tuning of gene expressions have been reported as a potential etiology for schizophrenia in many other genes (for example, Itokawa et al 2003a, 2003b).

Linkage of schizophrenia to chromosome 15q was reported by several groups, but the markers giving peak lods in different studies span over 60 Mb of the genome: D15S976 at 29.3 Mb (http://genome.ucsc.edu/) (Liu et al 2001), D15S652 at 90.3 Mb (Park et al 2004), D15S1010 at 30.9 Mb (Riley et al 2000) and D15S117 at 56.3 Mb (Williams et al 1999). The promising candidates on 15q include cholinergic receptor, muscarinic 5 (at

```
                                                  -1126T>C (SNP1)
aattatctaattccagtaaaacttaatggggcccttcccacccctggcca
                                                  CCAAT
atggttggtgtgaaatgtaatttgacttaaactgtctaaggctgtgaaag

tgtacaatattcggaagggtgggggcattaccccttgtgggagccatat

ttttctagaaggcattttgatcaagacaggcctcccgcggttattgatct

tagggtcattgagagtccaagaactggggagatgaaggccacccggcgtc
           -907G>C (SNP2)   MZF1                   CREB
agcaccgcgagggcggagggaaggcgggcgctgagtctcccattggggtt
                  -851T>C (SNP3)                NF-Y
ctagccgagggttccagccaggattgcgtgatctgggggcagctcggaga
                   GATA-1-2   GATA-1
gctctcccgacccagtatcgggtctataaatggagataaagatcagcacc
                          TATA      GATA-1/-2
                           TATA
                 .
                 .
                 .
                 .
gcgcggagccgggcgcggggcgggggaagggagaggagggagggaggggc
                    Sp1
aggcgggagccgggagggagcgagcgggctcgccgcgcctgagcaacccc

tgcctgtcgctgccgctgccgctgccgctgccgccgccggcccggactcg

tccggagcgcagggtgtctgcccagctgcgcgcggcgcgcggaggctccg
                                             -27G>T (SNP4)
gcgtccgccgctgcgccctccggcccctgctcctcgcgccggcccgcgtg
 -19C>T (SNP5)
GGTCCCGGCGGGCGCGAACCCACCATGCAGCTGCAGTTCCGGAGCTGGAT

GCTGGCCGCGCTCACGCTGCTCGTGGTCTTCCTCATCTTCGCAGACATCT

CAGAGATCGAAGAAGAAATCGGgtaaatagctgctcccaggcccgtgcca
```

Figure 3. Nucleotide sequence of the *SIAT8B* promoter region. The sequences for the promoter region and intron 1 are shown in lowercase and those of the first exon, in uppercase. The consensus binding sites and locations of single nucleotide polymorphisms (SNPs) are denoted. The initiation codon ATG is boxed.

32.1 Mb) (De Luca et al 2004) and cholinergic receptor, nicotinic, alpha polypeptide 7 (at 30.2 Mb) (Freedman et al 2001), in addition to the current *SIAT8B* (at 90.8 Mb). It would be an important theme to examine whether each linkage signal on 15q can be explained by a single (different) gene or combination of genes.

The current results may be relevant to hippocampal dysfunction in schizophrenia. *SIAT8B* transcripts are abundant in fetal brain and are present predominantly in the hippocampus of adult brain (Angata et al 1997). The PSA-NCAM1-positive newborn cells migrate into the granular cell layer from the subgranule zone in the adult hippocampus (Nakagawa et al 2002). Elevated level of *SIAT8B* could perturb the size and/or structure of PSA complexes thereby disrupting interactions between newly generated neurons and surrounding cells resulting in aberrant hippocampal formation.

We detected one schizophrenic patient with the Glu141-to-Lys141 missense mutation who belonged to a multiply affected large pedigree but DNA samples from other family members were not available for analysis. No other schizophrenic or control subjects carried this variant. The catalytic sialyl motifs L and S, and the C-terminal region are important for the enzymatic activity of *SIAT8B* (Angata et al 2001). The Glu141Lys polymorphism is located near the sialyl motif L and elicits a protein charge change (Scheidegger et al 1995). An examination of the functional consequence of the Glu141-to-Lys141 substitution would be interesting.

In conclusion, further replication studies are warranted using larger size of samples. It would be intriguing to examine the correlation between *SIAT8B* genotypes and PSA content in the brain, and to further investigate the biological impact of neuronal

polysaccharides on the pathogenesis of schizophrenia in a molecular genetic approach.

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Analysis of correlation between serum D-serine levels and functional promoter polymorphisms of *GRIN2A* and *GRIN2B* genes

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Abstract

D-Serine is an endogenous coagonist that increases the opening of *N*-methyl-D-aspartate (NMDA)-type glutamate receptor channels. We previously reported a reduction of D-serine serum levels in schizophrenia, supporting the disease hypothesis of NMDA receptor-mediated hypo-neurotransmission. The serum levels of D-serine are thought to reflect brain D-serine content. It is important to understand whether there is a direct link between the altered D-serine levels and NMDA receptor expression in vivo or whether these are independent processes. Two polymorphisms are known to regulate the expression of NMDA receptor subunit genes: $(GT)_n$ (rs3219790) in the promoter region of the NR2A subunit gene (*GRIN2A*) and −200T>G (rs1019385) in the NR2B gene (*GRIN2B*). These polymorphisms are also reported to be associated with schizophrenia. Therefore, we examined the correlation between these two polymorphisms and D-serine serum levels in mentally healthy controls, schizophrenics and the combined group. We observed no significant genotype–phenotype correlations in any of the sample groups. However, analyses of larger sample numbers and the detection of additional polymorphisms that affect gene expression are needed before we can conclude that NMDA receptor expression and serum levels of D-serine, if involved in schizophrenia pathophysiology, are independent and additive events.
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Keywords: NMDA receptor; NR2A subunit; NR2B subunit; Short tandem repeat; SNP; Schizophrenia

Schizophrenia is a chronic and disabling mental illness of unknown etiology. Among the proposed mechanisms, a disturbance in *N*-methyl-D-aspartate (NMDA) receptor-mediated neuronal transmission offers a cogent hypothesis for schizophrenia development. This hypothesis is based on clinical observations that phencyclidine and its congener anesthetic ketamine, both of which act as non-competitive antagonists of the NMDA receptor, provoke positive and negative schizophrenia-like symptoms in healthy controls. Additionally, phencyclidine exacerbates schizophrenic symptoms in patients [9,10]. We previously identified a variable $(GT)_n$ repeat (rs3219790) in the 5′-regulatory region of *GRIN2A*, a gene encoding the NR2A subunit of the NMDA receptor complex. We demonstrated that this repeat sequence repressed transcriptional activity in a length-dependent manner, with longer repeats inducing greater repression of the promoter [7]. These data and our detection of a significant association of the repeat polymorphism with schizophrenia, and an over-representation of longer alleles in schizophrenic patients, support the NMDA receptor disease theory [7,8]. Functionally distinct NMDA receptor subtypes are formed by heteromeric assembly of NR1 and one of either the NR2 (NR2A–D) or NR3 (NR3A and B) subunits [2,13]. Miyatake et al. [12] identified a functional single nucleotide polymorphism (SNP), −200T>G (rs1019385), in the promoter region of the NR2B subunit gene (*GRIN2B*), and reported that the transcriptionally less competent G allele was significantly more prevalent in schizophrenics than controls. To date, no other polymorphisms that regulate the expression of NMDA receptor subunit genes have been recorded.

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