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Relationship between three serotonin receptor subtypes (*HTR3A, HTR2A* and *HTR4*) and treatment-resistant schizophrenia in the Japanese population

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Abstract

The proportion of treatment-resistant schizophrenia (TRS) has been estimated as 20–40% in the schizophrenic patients. Genetic factors are considered to be involved in the development of this condition. Serotonin subtypes are hypothesized to be the candidate genes. In the present study, single marker and haplotype analyses between several mutations of serotonin receptor subtypes (*HTR2A*, *HTR3A* and *HTR4*) and TRS (TRS = 101, NON-TRS = 239) were performed to determine a possible relationship with the development of TRS. Additionally, we also compared the daily neuroleptic dosage among each genotype. No significant association was observed between TRS and each allele, genotype, and haplotype. However, the daily neuroleptic dosage that patients had been receiving during their maintenance therapy was significantly higher in patients with the T/T genotype of *HTR3A* polymorphism (rs1062613, $p = 0.041$). The present results support further research to examine the relationship between *HTR3A* polymorphism and the development of TRS in the Japanese population.

Keywords: *HTR*; Serotonin; Antipsychotic drug; Refractory; Gene; Haplotype

The proportion of treatment-resistant schizophrenia (TRS) has been estimated as 20–40% in the schizophrenic patients, and this unfortunate situation in the clinical psychiatric field still remain unchanged even after the introduction of several atypical antipsychotic agents [27]. Among the atypical antipsychotics, only clozapine has been reported to be effective for 30–60% of schizophrenic patients refractory to typical and atypical antipsychotics [20,28]. Clozapine is known to provide antipsychotic effects through binding to the several serotonin receptor subtypes (5-HT) [3] although the actual mechanism of clozapine for TRS has not been elucidated yet. In order to clarify this mechanism several researches investigated the predictable genetic factors for the clinical response to clozapine, as a result a significant association with the 5-HT receptor subtypes has been reported in a number of studies as follows.

Clozapine has a high affinity for 5-HT2A receptor [21] and produces a significant downregulation of cortical 5-HT2A receptor in the radioligand binding studies [2]. In addition, two PET studies have shown that the systemic administration of clozapine to schizophrenic patients produces an 84–90% occupation of cortical 5-HT2A receptor [23,6]. A couple of researches have reported the association between 5-HT2A receptor gene (*HTR2A*) polymorphism and TRS [13,7] or response to clozapine [17], although no association study has been reported in the Japanese subjects with TRS.

Since the 5-HT3A receptor has been reported to have potential anxiolytic and antipsychotic properties from animal studies, 5-HT3A receptor antagonists are being explored as therapeutic agents for a variety of behavioral disorders [5]. Additionally, 5-HT3A receptor gene (*HTR3*) is located on 11q23.1, where linkage with schizophrenia has been suggested in several studies [19,16]. These results suggest that *HTR3* may be related to

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the treatment response in the schizophrenic patients. Gutierrez have reported no association between *HTR3A* polymorphism and clozapine response [8], however, this study did not take haplotype block structure into consideration and did not cover whole genomic region of *HTR3A*.

5-HT4 receptor gene (*HTR4*) also has been reported to be associated with schizophrenia in the Japanese population [25]. Therefore, this gene could also be a candidate gene for TRS.

Thus, the *HTR2A*, *3A*, *4* could be considered as plausible genes related to the development of the TRS. Therefore, in the present study, we performed linkage disequilibrium (LD) analysis of *HTR3A*, followed by the case-control association studies between *HTR3A* polymorphisms and TRS using single-marker association analyses and haplotype analyses. In addition, the association was also examined among *HTR2A* polymorphism, *HTR4* polymorphism and TRS.

This study was initiated after the approval by the Ethics Committee of the Nagoya University School of Medicine. Written informed consent was obtained from all subjects at study entry.

A total of 340 patients with schizophrenia (male = 200; female = 140; age: 54 ± 12.8; duration of illness: 33.6 ± 12.4 years; daily neuroleptic dosage: 1021 ± 1857 mg/day) who had been diagnosed using the criteria of DSM-IIIR [1] were selected in this study. All patients were Japanese descent and had been hospitalized and receiving antipsychotic drugs for more than 1 year.

The definition of TRS is described elsewhere in the previous study [11]. Briefly, information about the neuroleptic therapy that the schizophrenic patients had been receiving was obtained from their clinical records. The daily neuroleptic dosage was calculated from the recent 1-year neuroleptic prescription history. Schizophrenic patients were diagnosed as having TRS when they had been hospitalized for more than 1 year and had been receiving antipsychotic therapy at dosages of at least 1000 mg/day chlorpromazine equivalents for more than 1 year.

Using the information obtained from the HapMap Database and the dbSNP Database, two single nucleotide polymorphisms – rs1062613 and rs1176713 – were selected as haplotype tag SNPs (htSNPs) that covered the whole coding region, 5′ flanking region upstream 500 bp, and 3′ UTR region downstream 500 bp of *HTR3A*. The LD block was defined using HAPLOVIEW Version 3.0 (http://www.broad.mit.edu/mpg/haploview/) as a region of $D' > 0.8$. In each LD block, haplotype frequency was estimated by the expectation-maximization (EM) algorithm and htSNPs were selected using the same program. Additionally, a SNP (rs6313) of *HTR2A* and two SNPs (rs2278392, rs3734119) of *HTR4* which have been reported to be associated with schizophrenia in the previous study [25] were selected. Genotyping was carried out using polymerase chain reaction-restriction fragment length polymorphism assays or direct sequence assays for each SNP. Sequences of each primer pairs are available on request.

Genotype deviation from the Hardy–Weinberg equilibrium (HWE) was evaluated by Chi-square test. Single-marker and haplotype analyses were performed using SPSS Version 11.0J (Tokyo, Japan) and Cocaphase 2.403 (http://www.rfcgr.mrc.ac.uk/~fdudbrid/software/unphased/), respectively. Comparison of the daily neuroleptic dosage among each genotype was performed using Mann–Whitney *U*-test. Power calculation was performed by Power Calculator (http://calculators.stat.ucla.edu/powercalc/). The level of significance for all statistical tests was set at 0.05.

A total of 101 schizophrenic patients were identified as the TRS (TRS: male = 67, female = 34, age = 50 ± 10.5, onset age = 20 ± 5.3; NON-TRS: male = 133, female = 106, age = 56 ± 13.1, onset age = 23.5 ± 8.2). The male ratio tended to be higher in the TRS patients ($p < 0.1$), and the age at onset was significantly younger in this group ($p = 0.009$). However, no significant difference was observed in the incidence of any psychiatric symptom between the two groups, such as delusion and hallucination, bizarre behavior, disorganization, and negative symptoms at their first episode, as reported in our previous report [12]. The genotype distributions of the polymorphisms did not deviate significantly from the HWE in each study group for any polymorphism. The genotype and allele frequencies of three kinds of serotonin receptor genes in TRS and NON-TRS groups are shown in Table 1.

No significant association was observed in the single marker analysis of *HTR2A*, *HTR3A*, and *HTR4*, and in haplotype analysis of *HTR3A* and *HTR4* (Table 1).

The characteristics of neuroleptic treatment among the three subgroups showing each SNP polymorphism are shown in Table 2. In rs1062613 of *HTR3A*, the daily neuroleptic dosage during maintenance therapy was significantly higher in patients with the T/T genotype than the others ($p = 0.041$).

When the proportion of TRS was set to be 30% [9], we obtained more than 80% power to detect an association with the SNPs of which the minor frequency is more than 10%.

The results presented here suggest that *HTR3A* may be involved in the development of TRS in the Japanese population. In this study, significant difference in the daily neuroleptic dosage received during maintenance therapy was observed in schizophrenic patients with the T/T genotype of *HTR3A* polymorphism (rs1062613).

The SNP rs1062613 is located on the promoter region of *HTR3A* and has been reported to regulate the expression of this gene [22]. Since presynaptic 5-HT3A receptors modulate the release of several neurotransmitters in various brain regions [15,26], the abnormal expression of *HTR3A* might increase the dopamine concentration in the synaptic cleft. This may lead to increase the therapeutic antipsychotic doses in the schizophrenic patients with this mutation.

Additionally, several antipsychotic drugs reduce the dopaminergic neurotransmission by antagonizing the 5-HT3A receptor [24]. Therefore, reduction in the expression of 5-HT3A receptor may weaken the therapeutic effect of antipsychotics through this pathway; even higher dose of most antipsychotic drugs may not reduce the dopaminergic neurotransmission.

Furthermore, this SNP has been reported to have a critical role in the amygdala activity leading to the facial expression recognition [10], and the defect of facial expression recognition has been reported to be a specific symptom to the schizophrenia including TRS [4,18]. Therefore, this SNP may have a role in

Table 1
Genotype and allele frequencies of *HTR2A*, *HTR3A* and *HTR4* polymorphisms in TRS and NON-TRS

	Genotype			*p*-Value	Allele frequency (%)		*p*-Value	Global *p*-value
HTR2A								
rs6313	C/C	C/T	T/T		C	T		
TRS	19	58	23	0.500	48	52	0.777	
NON-TRS	48	123	68		46	54		
HTR3A								
rs1062613	C/C	C/T	T/T		C	T		
TRS	75	21	5	0.117	85	15	0.400	
NON-TRS	189	47	3		89	11		0.576
rs1176713	A/A	A/G	G/G		A	G		
TRS	49	38	14	0.744	67	33	0.648	
NON-TRS	124	86	27		70	30		
HTR4								
rs2278392	G/G	G/A	A/A		G	A		
TRS	59	36	7	0.867	76	24	0.868	
NON-TRS	148	80	15		77	23		0.863
rs3734119	T/T	T/C	C/C		T	C		
TRS	59	36	8	0.891	75	25	0.869	
NON-TRS	148	80	19		76	24		

the development of TRS based on the effect of the SNP on the impairment of facial expression recognition.

The definition of TRS in the present study is different form that proposed by Kane et al. [14]. Since the polypharmacy is widely prevalent in the antipsychotic treatment of schizophrenia in Japan. In the present study, the psychopathology of TRS was defined by the total antipsychotic doses that the schizophrenic patients had been receiving during the recent 1 year, that is, the severity of illness was extrapolated by the total antipsychotic doses. In addition, they had been hospitalized for more than 1 year, indicating that they had been no good level of functioning over this period. In fact, age at disease onset had been observed to be significantly younger in the TRS subjects, suggesting that the younger onset patients tend to less response to the antipsychotic therapy. Therefore, we consider that virtually no essential difference exists between the present definition of TRS enrolled in Japan and that proposed by Kane et al. [14].

Table 2
Characteristics of NLP treatment among three subgroups showing *HTR2A*, *HTR3A* and *HTR4* polymorphisms

	Genotype		
HTR2A			
rs6313	C/C	C/T	T/T
Daily NLP	575 (2–4042)	603 (4–12893)	372 (3–6283)
HTR3A			
rs1062613	C/C	C/T	T/T
Daily NLP	496 (2–12893)	568 (5–12850)	1179 (281–3048)[a]
rs1176713	A/A	A/G	G/G
Daily NLP	559 (3–8337)	417 (2–12893)	710 (42–4226)
HTR4			
rs2278392	G/G	G/A	A/A
Daily NLP	491 (2–12893)	600 (4–6283)	460 (50–2262)
rs3734119	T/T	T/C	C/C
Daily NLP	491 (2–12893)	605 (4–6283)	439 (30–2262)

Data are expressed as median (min–max).
[a] $p = 0.041$ when compared to the (C/C + C/T) subgroup.

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No Association Between the Protein Tyrosine Phosphatase, Receptor-Type, Z Polypeptide 1 (*PTPRZ1*) Gene and Schizophrenia in the Japanese Population

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NRG1-ERBB signaling influences the risk for schizophrenia pathology. A recent study has reported that *MAGI1*, *MAGI2*, and protein tyrosine phosphatase, receptor-type, Z polypeptide 1 (*PTPRZ1*; located on 7q31.3) gene products regulate the NRG1-ERBB4 signaling pathway, and *PTPRZ1* is associated with schizophrenia in a Caucasian population. By applying a gene-based association concept, we analyzed any association between *PTPRZ1* tagging SNPs and schizophrenia in the Japanese population (576 schizophrenics and 768 controls). After linkage disequilibrium analysis, 29 single nucleotide polymorphisms (SNPs) were genotyped using a 5′-exonuclease allelic discrimination assay. We found a significant association of one tagging SNP in a genotype-wise analysis ($P = 0.007$); however, this might be resulted from type I error due to multiple testing ($P = 0.17$ after SNPSpD correction). No association was observed between schizophrenic patients and controls in either allelic, genotypic, or haplotypic analyses. Our results therefore suggest that *PTPRZ1* is unlikely to be related to the development of schizophrenia in the Japanese population.

KEY WORDS: association study; *NRG1*; *ERBB4*; linkage disequilibrium; HapMap

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INTRODUCTION

Schizophrenia is a chronic and devastating psychiatric disorder with a global morbidity risk of approximately 1%. While schizophrenia is highly heritable (heritability score of approximately 0.8), the underlying genetics are complex, and the interpretation of genetics data has proven difficult [Freedman 2003; Ross et al. 2006]. The hypothesis, which this disease is a developmental disorder of the nervous system with a late onset of characteristic symptoms, has been gaining acceptance over the past years, and several candidate predisposition genes such as neuregulin-1 (*NRG1*), disrupted-in-schizophrenia 1 (*DISC1*), dysbindin (*DTNBP1*), and glutamate decarboxylase 67 (*GAD1*) have been reported [Lewis and Levitt, 2002; Rapoport et al., 2005; Harrison, 2007].

Among these genes, *NRG1* is regarded as one of the most promising susceptibility genes for schizophrenia [Stefansson et al., 2002; Li et al., 2006; Munafo et al., 2006]. NRG1-ERBB signaling may contribute to the pathogenesis of schizophrenia by affecting neuronal migration, cortical connectivity, neurotransmitter receptor expression (NMDA and $GABA_A$), oligodendrocyte development, and myelination [Norton et al., 2006; Li et al., 2007; Woo et al., 2007]. Any associated alterations of NRG1-ERBB signaling would thus support a neurodevelopmental and a glutamate/GABA hypothesis of schizophrenia causation [Corfas et al., 2004].

Recently, MAGI proteins were identified as substrates for the *ERBB4* gene product by both a yeast two-hybrid analysis and a kinase assay [Montgomery et al., 2004]. Likewise, MAGI proteins are dephosphorylated by receptor protein tyrosine phosphatase beta (RPTPβ) [Fukada et al., 2005], and thus notably, RPTPβ may also regulate the NRG1-ERBB4 signaling [Buxbaum et al., 2007].

RPTPβ is the *PTPRZ1* (protein tyrosine phosphatase, receptor type, Z polypeptide 1) gene product, and RPTPβ protein is highly expressed during embryogenesis as a transmembrane protein, primarily in the central nervous system [Levy et al., 1993]. Tyrosine phosphorylation and dephosphorylation play a key role in the signaling of cell growth and differentiation, and the *PTPRZ1* gene product is believed to play a role in the recovery and survival of oligodendrocytes in demyelinating disease [Harroch et al., 2002].

In the aforementioned NRG1-ERBB4 study, a case-control association study between *PTPRZ1* and schizophrenia has been carried out, and *PTPRZ1* has been demonstrated to be associated with schizophrenia in a United Kingdom case-control cohort [Buxbaum et al., 2007]. However, while *PTPRZ1* is considered to be one of the plausible candidate genes for schizophrenia, replication of this positive association is required in order to demonstrate that *PTPRZ1* is a true susceptibility gene for this disease.

It is widely accepted that there are certain limitations in replicating a genetic association study using the same or a smaller number of single nucleotide polymorphisms (SNPs) as the original investigation. One major limitation is due to differences in allele frequency or variations of the linkage disequilibrium (LD) structure (population dependence) among each ethnicity. To overcome this limitation, a gene-based approach, rather than a SNP-based or haplotype-based approach, is currently recommended [Neale and Sham, 2004]. In such studies, it is important to include both the gene as well as the gene-flanking regions when testing for any associations, and it is also important to select genetic variants which adequately reflect the LD background by the standardized disequilibrium coefficient (D′) and squared correlation coefficient (r^2) in the targeted population (e.g., tagging SNPs). By applying this gene-based association approach, we sought to determine the association, if any, between *PTPRZ1* tagging SNPs and schizophrenia in the Japanese population.

MATERIALS AND METHODS

Subjects

The cohorts used in this study consisted of 576 patients with schizophrenia (341 males and 235 females, mean age ± SD = 50.1 ± 15.1 years) and 768 healthy control subjects (365 males and 403 females, mean age ± SD = 40.1 ± 15.6 years). All subjects were unrelated to each other and were of Japanese ethnicity. The patients were all diagnosed according to the Diagnostic and Statistical Manual of Mental Disorders, Fourth Edition, Text Revision (DSM-IV-TR) criteria for schizophrenia, with consensus reached among at least two experienced psychiatrists on the basis of unstructured interviews as well as a review of the subjects' medical records. All healthy control subjects were also psychiatrically screened with brief diagnostic unstructured interviews. Subjects who had current or past contact with psychiatric services were excluded. After describing the study to each subject, written informed consent was obtained. This study was approved by the Ethics Committee of the Nagoya University Graduate School of Medicine.

Tagging SNP Selection

To analyze genetic association, we implemented a gene-based approach. This method implies the inclusion of both the gene region and the gene-flanking regions in the association study [Neale and Sham, 2004]. The *PTPRZ1* gene contains 30 exons spanning approximately 188900 base pairs (bp), and three other splicing isoforms have been reported thus far [Garwood et al., 2003; Paul and Lombroso, 2003].

We first consulted the HapMap database (release#22; phase2, April 2007, population: Japanese in Tokyo, minor allele frequency (MAF): more than 0.05). All SNPs in the entire gene region covering all isoforms, as well as the 5,000 bp upstream 5′ flanking region and the 5,000 bp downstream 3′ flanking region, were listed.

Then we defined 28 tagging SNPs (Table I) with the criterion of an $r^2 > 0.8$ in 'pair-wise tagging only' mode using the 'Tagger' program, implemented by Haploview software version 4.0 (http://www.broad.mit.edu/mpg/haploview/index.php) [Barrett et al., 2005; de Bakker et al., 2005], considering two points in particular. First, we mandatorily included marginal and significant SNPs reported in previous study (Table I; rs6466808, rs10278079, rs1196471, rs2693657, rs1147502, rs1147497, rs1147489, and rs1206381; indicated by asterisk) as tagging SNPs, except for rs1196513 and rs13241278 [Buxbaum et al., 2007]. Because rs1196513 and rs13241278 were not listed in the HapMap database, we could not analyze the LD patterns of these SNPs. Second, due to unavailability of the reliable genotyping method for rs1206384, we genotyped rs1860721 (No.8) instead, whose r^2 value with rs1206384 was 1 (HapMap data). There were three validated exonic SNPs (MAF > 0.05) and some SNPs in the 3′ or 5′ flanking of the gene, and all these SNPs were covered by the selected tagging SNPs.

Of two other significant SNPs reported by Buxbaum et al. (rs1196513 and rs13241278), rs13241278 was included, but rs1196513 was not included in our analysis because this SNP was not validated and its frequency was not reported in the dbSNP database (NCBI). Overall, 29 SNPs were examined.

SNP Genotyping

Venous blood was drawn from each subject and genomic DNA was extracted from whole blood according to standard procedures. Genotyping of all tagging SNPs was carried out using a TaqMan 5′-exonuclease allelic discrimination assay (Applied Biosystems Japan Ltd., Tokyo, Japan). TaqMan probes and Universal PCR Master Mix were obtained from Applied Biosystems. Allelic-specific fluorescence was measured using the ABI PRISM 7900 Sequence Detector System (Applied Biosystems). Details regarding reagents or reaction conditions are available upon request.

Statistical Analysis

Genotype deviation from the Hardy–Weinberg equilibrium (HWE) and single-marker allelic association were evaluated using Haploview. Analysis of linkage disequilibrium between markers (r^2 and D′) was also performed using Haploview. Genotypic association was tested by the chi-squared test or by Fisher's exact test. Genotypic association of SNPs that deviated from HWE was analyzed using the Cochran-Armitage trend test for a multiplicative model of inheritance [Balding, 2006]. For haplotype-wise analysis, LD blocks were initially defined in accordance with Gabriel's criteria using Haploview software. Haplotypic analyses were performed with Unphased software version 2.403 [Dudbridge, 2003], which performs log-likelihood ratio tests under a log-linear model for global P values. Rare haplotypes found in less than 3% of both case and control subjects were excluded from the association analysis to provide greater sensitivity and accuracy when the effect was seen in common haplotypes, and the expectation-maximization algorithm was then employed. rs13241278 was excluded from haplotypic analysis because this SNP was not selected by Tagger. The significance level for all statistical tests was $P < 0.05$. Power calculations were performed using the genetic statistical package Genetic Power Calculator [Purcell et al., 2003] (http://pngu.mgh.harvard.edu/~purcell/gpc/). The number of effective independent SNPs assayed was estimated by the spectral decomposition method of Nyholt using SNPSpD software. This software is able to reflect the correlation of markers (LD) on corrected P values to control for inflation of the type I error rate [Nyholt, 2004].

RESULTS

The genotype and allele frequencies of each SNP from schizophrenic patients and healthy control subjects are summarized in Table I. The observed genotype frequencies of two tagging SNPs deviated from HWE (rs1206477 and rs13241278). The LD relationships between markers are provided in Table II. The LD patterns observed in our controls were nearly identical to those of the JPT HapMap samples, but obviously different from those of the CEU HapMap samples. Only rs1196511 showed a significant association with schizophrenics in a genotype-wise analysis ($P = 0.007$). However, this

TABLE I. Association Analysis of Twenty-Nine SNPs of *PTPRZ1*

No.	SNP ID		M/m	N		CON			SCZ			MAF		Genotype *P*-value	Corrected *P*-value	Allele *P*-value[d]	LD block[d]	GRR[e]
				CON	SCZ	M/M	M/m	m/m	M/M	M/m	m/m	CON	SCZ					
1	rs13437930	Intron1	G/C	762	566	559	179	24	399	150	17	0.15	0.16	0.45[a]		0.34	/	1.34
2	rs2402593	Intron1	A/G	762	564	400	303	59	272	239	53	0.28	0.31	0.25[a]		0.10	I	1.27
3	rs1916885	Intron1	T/C	762	565	326	337	99	255	240	70	0.35	0.34	0.69[a]		0.43	I	1.26
4	rs3757548	Intron1	T/C	758	566	664	91	3	499	65	2	0.06	0.06	0.93[b]		0.75	I	1.50
5	rs1206477	Intron1	C/G	761	566	361	307	93	266	242	58	0.32	0.32	0.68[c]		0.68	I	1.26
6	rs6974265	Intron1	A/G	761	566	578	171	12	410	150	6	0.13	0.14	0.19[b]		0.26	I	1.36
7	rs1011692	Intron1	T/C	761	564	443	272	46	318	220	26	0.24	0.24	0.31[a]		0.91	I	1.29
8	rs1860721	Intron2	C/T	761	566	582	164	15	445	118	3	0.13	0.11	0.07[b]		0.16	II	1.36
9	rs4731044	Intron2	C/G	762	563	323	327	112	238	256	69	0.36	0.35	0.39[a]		0.54	II	1.26
10	rs6466808*	Intron2	G/A	759	565	356	323	80	255	238	72	0.32	0.34	0.45[a]		0.28	II	1.26
11	rs1196482	Intron2	C/T	761	566	495	237	29	356	193	17	0.19	0.20	0.42[a]		0.67	II	1.31
12	rs10278079*	Intron2	A/G	762	566	392	295	75	268	235	63	0.29	0.32	0.32[a]		0.14	II	1.27
13	rs1196511	Intron2	G/A	761	565	191	358	212	142	306	117	0.51	0.48	0.01[a]	0.17[f]	0.07	II	1.25
14	rs3817483	Intron6	T/C	762	566	592	158	12	443	111	12	0.12	0.12	0.69[a]		0.99	II	1.37
15	rs10225212	Intron8	G/T	761	566	521	213	27	363	175	28	0.18	0.20	0.18[a]		0.06	/	1.32
16	rs1196471*	Intron8	T/C	762	564	316	349	97	230	261	73	0.36	0.36	0.97[a]		0.81	III	1.26
17	rs2693657*	Intron9	G/A	762	566	316	350	96	230	265	71	0.36	0.36	0.95[a]		0.84	III	1.26
18	rs1196474	Intron10	G/A	761	566	379	304	78	273	236	57	0.30	0.31	0.81[a]		0.70	III	1.37
19	rs17144005	Intron11	A/G	762	565	667	91	4	508	55	2	0.07	0.05	0.38[b]		0.17	III	1.50
20	rs1147502*	Intron13	T/G	762	565	330	337	95	249	254	62	0.35	0.34	0.71[a]		0.54	III	1.26
21	rs12670616	Intron13	T/G	762	565	697	63	2	524	42	0	0.04	0.04	0.53[b]		0.32	III	1.61
22	rs1147497*	Intron14	T/G	761	565	313	357	91	232	265	68	0.35	0.36	1.00[a]		0.97	III	1.26
23	rs1147492	Intron18	C/T	762	563	263	364	135	183	272	108	0.42	0.43	0.67[a]		0.37	III	1.25
24	rs1147491	Intron18	A/G	762	565	457	257	48	341	203	21	0.23	0.22	0.10[a]		0.37	III	1.29
25	rs1918031	Intron18	G/A	761	566	532	203	26	408	146	12	0.17	0.15	0.33[a]		0.23	III	1.32
26	rs1147489*	Intron19	A/G	761	566	506	236	19	372	174	20	0.18	0.19	0.54[a]		0.55	III	1.32
27	rs1147488	Intron22	A/G	762	566	571	174	17	436	122	8	0.14	0.12	0.46[b]		0.27	III	1.35
28	rs1206381*	Intron27	A/C	761	566	322	348	91	249	253	64	0.35	0.34	0.82[a]		0.53	III	1.26
	rs13241278*	Intron8	C/T	759	565	385	275	99	280	223	62	0.31	0.31	0.81[c]		0.80		1.26

N, number; M, major allele; m, minor allele; CON, control; SCZ, schizophrenia; MAF, minor allele frequency; GRR, genotype relative risk ($\alpha = 0.05$, $1 - \beta = 0.8$).
IDs with asterisk (*) represent significant or marginally significant SNPs in Buxbaum's report.
Genotypic *P*-value was calculated by the chi-squared test (a), Fisher's exact test (b), and the Cochran–Armitage trend test(c).
Allelic *P*-value was calculated and LD block (Gabriel's criteria) was defined using Haploview software (d).
GRR was calculated using Genetic power calculator (e).
Corrected *P*-value was calculated by Nyholt's method (f).
Effective number of independent marker loci [Meff]: 24.8646.
Experiment-wide significance threshold required to keep Type I error rate at 5%: 0.002.

significance may be the result of a type I error due to multiple testing. We performed *P*-value correction by using the SNPSpD program (the effective number of independent marker loci: 24.8646; the experiment-wide significance threshold required to keep type I error rate at 0.05: 0.002; $P = 0.17$ after SNPSpD correction) [Nyholt, 2004]. No association was observed between the schizophrenic patients and their controls in allelic, genotypic, or haplotypic analyses (Tables I and III). Greater than 80% power in detecting any association with schizophrenia was obtained when the genotype relative risk (GRR) was set between 1.25 and 1.61 under a multiplicative model of inheritance assuming the disease prevalence to be 1% and the population susceptibility allele frequencies to be the values observed in control samples.

DISCUSSION

The 'common disease-common variant' hypothesis postulates that linkage disequilibrium should be detected by the haplotype association test if the risk haplotype is linked to causal variants for disease [Chakravarti, 1999]. Regarding the Japanese population, therefore, the data presented in this article do not provide sufficient evidence for the involvement of the *PTPRZ1* gene in conferring susceptibility for schizophrenia.

In this study, we could not replicate a previous report [Buxbaum et al., 2007], which revealed a significant association between *PTPRZ1* and schizophrenia in a Caucasian population. The discrepancy between Japanese and Caucasian populations may derive from ethnic differences in the etiology of schizophrenia. Although the sample size used here is smaller than the sample size of the original study, we obtained a statistical power expected to detect any possible association, and so the possibility of a type II error is unlikely. The GRR value calculated using power analysis was appropriate when compared to other promising candidate genes for schizophrenia [Shifman et al., 2002; Schwab et al., 2003]. In this regard, a prompt gene-based replication study has become feasible as the International HapMap project progresses. In this case, however, *PTPRZ1* is a complicated gene with a transcript that is spliced into four distinct isoforms, and the HapMap database focused only one isoform (NM_002851). With such a limitation, other SNPs involved in mRNA splicing, while having attracted great attention in the pathology of schizophrenia [Law et al., 2007], might nonetheless be overlooked. Thus, it might be useful to investigate not only exons but also splice junctions of this gene.

Two additional limitations need to be addressed in order to discuss the present results. First, age-unmatched and male-to-female ratio unmatched cohorts were examined in the present study. The mean age of the controls is younger than that of the patients. This means that a number of these younger controls, though likely not more than eight subjects given a lifetime morbidity risk of 1%, may later develop schizophrenia. This confounding factor might weaken the power of the present study. We also performed exploratory analysis to investigate the effect of age and gender on the disease status. Based on the results of the analysis using logistic regression model, these variables did not seem to be involved in the results of present association study (data not shown). Secondly, another limitation, which must be addressed, is that other candidates related to the NRG1-ERBB signaling would also be in the locus heterogeneity. In this case, causal variants with extremely rare MAFs and allelic heterogeneity should be also considered. Likewise, the combined effect between SNPs on *PTPRZ1* and SNPs on the other genes (*ERBB4*, MAGIs, etc) might actually prove to be a stronger predisposition factor.

Moreover, the definition of phenotypes is vital for a genetic association study. Therefore, endophenotypes (being more

TABLE II. Linkage Disequilibrium Analysis of *PTPRZ1*

No.	SNP ID	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28
1	rs13437930		0.99	0.97	1.00	0.98	1.00	0.89	1.00	0.65	0.55	0.07	0.57	0.72	0.86	0.58	0.61	0.43	0.43	0.44	0.63	0.49	0.73	0.41	0.17	0.23	0.83	0.02	0.90	0.34
2	rs2402593	0.45		1.00	1.00	0.99	1.00	0.98	0.64	0.80	0.70	0.48	0.58	0.62	0.25	0.62	0.70	0.10	0.10	0.28	0.62	0.52	0.76	0.08	0.11	0.10	0.11	0.41	0.69	0.04
3	rs1916885	0.09	0.21		1.00	1.00	1.00	0.93	0.76	0.89	0.38	0.73	0.32	0.33	0.26	0.90	0.83	0.41	0.09	0.93	0.90	0.96	0.81	0.40	0.89	0.26	0.21	0.57	0.72	0.38
4	rs3757548	0.01	0.03	0.04		1.00	1.00	1.00	1.00	0.95	1.00	1.00	1.00	0.93	1.00	0.96	1.00	0.89	0.89	1.00	1.00	0.96	1.00	0.88	0.95	1.00	1.00	1.00	0.97	0.88
5	rs1206477	0.08	0.18	0.26	0.14		1.00	0.94	0.77	0.92	0.95	0.94	0.95	0.93	0.90	0.77	0.85	0.64	0.65	0.73	1.00	0.70	0.89	0.65	0.70	0.75	0.73	0.61	0.72	0.65
6	rs6974265	0.03	0.39	0.08	0.01	0.07		1.00	0.18	0.97	0.85	1.00	0.55	0.42	0.35	0.65	0.10	0.17	0.17	0.10	0.58	0.51	0.73	0.17	0.03	0.49	0.30	1.00	0.34	0.18
7	rs1011692	0.04	0.12	0.50	0.02	0.13	0.05		1.00	0.89	0.19	0.85	0.12	0.92	0.33	0.90	0.78	0.91	0.91	0.85	0.78	0.89	0.83	0.88	0.90	0.75	0.33	0.65	0.71	0.84
8	rs1869721	0.03	0.02	0.16	0.01	0.04	0.00	0.05		1.00	1.00	1.00	0.95	0.98	1.00	1.00	0.88	0.94	0.94	0.96	1.00	1.00	0.90	0.94	1.00	0.95	0.92	1.00	0.95	0.94
9	rs4731044	0.04	0.14	0.24	0.11	0.71	0.08	0.14	0.08		1.00	1.00	1.00	1.00	0.97	0.94	0.89	0.68	0.68	0.84	1.00	0.83	0.91	0.68	0.75	0.87	0.72	0.67	0.92	0.69
10	rs6466808	0.11	0.40	0.04	0.03	0.20	0.23	0.01	0.07	0.27		1.00	1.00	0.86	0.99	0.87	0.94	0.08	0.08	0.32	0.96	0.73	0.94	0.07	0.34	0.17	0.54	0.90	0.78	0.10
11	rs1196482	0.00	0.02	0.24	0.02	0.10	0.04	0.55	0.04	0.14	0.11		1.00	1.00	1.00	0.98	0.94	0.99	0.99	0.96	0.77	0.98	0.98	0.96	0.96	0.90	0.02	0.89	0.94	0.95
12	rs10278079	0.14	0.31	0.02	0.03	0.18	0.11	0.90	0.06	0.23	0.88	0.10		1.00	0.98	0.97	0.95	0.10	0.11	0.37	0.97	0.88	0.86	0.10	0.44	0.20	0.53	0.69	0.75	0.13
13	rs1196511	0.10	0.16	0.06	0.06	0.39	0.03	0.28	0.13	0.53	0.37	0.25	0.43		0.98	0.98	0.94	0.75	0.75	0.14	1.00	0.90	0.80	0.74	0.60	0.05	0.76	0.73	0.84	0.75
14	rs3817483	0.02	0.02	0.02	0.01	0.05	0.12	0.05	0.02	0.07	0.29	0.03	0.32	0.14		0.97	0.66	0.89	0.89	0.66	0.40	0.94	0.91	0.83	0.96	0.49	0.82	1.00	0.48	0.83
15	rs13241278	0.03	0.07	0.20	0.14	0.56	0.03	0.11	0.07	0.71	0.16	0.11	0.18	0.42	0.06		1.00	0.95	0.96	0.89	1.00	0.92	1.00	0.94	0.89	0.86	0.86	0.95	0.93	0.86
16	rs10225212	0.31	0.27	0.08	0.01	0.07	0.01	0.04	0.02	0.09	0.40	0.05	0.47	0.20	0.01	0.10		1.00	1.00	0.91	0.99	0.87	1.00	1.00	0.05	0.41	0.93	1.00	0.58	0.91
17	rs1196471	0.02	0.00	0.17	0.03	0.11	0.01	0.47	0.07	0.14	0.01	0.42	0.01	0.32	0.19	0.23	0.12		1.00	0.97	0.93	0.95	0.97	0.98	0.94	0.95	0.96	0.97	0.98	0.96
18	rs2693657	0.02	0.00	0.07	0.03	0.11	0.01	0.47	0.07	0.15	0.01	0.42	0.01	0.33	0.19	0.23	0.12	1.00		0.97	0.93	0.96	0.97	0.98	0.95	0.95	0.96	0.97	0.98	0.95
19	rs1196474	0.08	0.07	0.01	0.03	0.11	0.00	0.10	0.31	0.17	0.09	0.10	0.13	0.01	0.03	0.16	0.41	0.23	0.23	0.03	0.16	1.00	1.00	1.00	0.46	0.98	0.97	1.00	0.98	0.95
20	rs17144005	0.16	0.07	0.03	0.01	0.03	0.00	0.01	0.01	0.04	0.14	0.01	0.16	0.07	0.00	0.03	0.32	0.03	0.03	0.23	0.16	1.00	1.00	1.00	0.96	0.98	0.88	1.00	1.00	0.86
21	rs1147602	0.02	0.06	0.23	0.12	0.45	0.02	0.13	0.08	0.64	0.13	0.12	0.17	0.41	0.06	0.73	0.09	0.27	0.27	0.23	0.04	1.00	1.00	1.00	0.98	0.97	0.98	1.00	0.97	0.94
22	rs12670616	0.00	0.01	0.06	0.00	0.02	0.00	0.10	0.01	0.02	0.09	0.01	0.09	0.03	0.28	0.02	0.01	0.08	0.08	0.02	0.00	0.02	1.00	1.00	1.00	1.00	0.98	0.98	0.71	1.00	0.97
23	rs1147497	0.02	0.00	0.16	0.03	0.11	0.01	0.44	0.07	0.14	0.00	0.42	0.01	0.31	0.17	0.22	0.12	0.94	0.95	0.24	0.04	0.29	0.08	0.98	0.99	0.99	0.99	1.00	0.97	0.96
24	rs1147492	0.00	0.00	0.31	0.09	0.33	0.00	0.18	0.10	0.45	0.04	0.16	0.06	0.24	0.09	0.61	0.00	0.35	0.35	0.07	0.05	0.71	0.03	0.37	1.00	1.00	1.00	1.00	1.00	0.98
25	rs1147491	0.03	0.01	0.04	0.02	0.08	0.01	0.05	0.43	0.13	0.02	0.06	0.03	0.00	0.01	0.10	0.12	0.15	0.15	0.67	0.22	0.15	0.01	0.16	0.22	1.00	1.00	1.00	1.00	0.96
26	rs1918631	0.02	0.01	0.02	0.01	0.05	0.07	0.07	0.03	0.06	0.13	0.00	0.14	0.12	0.45	0.07	0.04	0.34	0.34	0.08	0.01	0.10	0.22	0.36	0.14	0.06	0.94	1.00	0.99	0.99
27	rs1147489	0.00	0.01	0.13	0.02	0.04	0.03	0.30	0.03	0.06	0.08	0.59	0.07	0.13	0.03	0.09	0.05	0.38	0.38	0.10	0.02	0.12	0.01	0.40	0.16	0.07	0.04		1.00	0.99
28	rs1147488	0.02	0.03	0.15	0.01	0.04	0.00	0.03	0.83	0.06	0.05	0.03	0.04	0.10	0.01	0.06	0.01	0.08	0.08	0.35	0.01	0.08	0.01	0.08	0.11	0.52	0.03	0.04		1.00
29	rs1206581	0.01	0.00	0.15	0.03	0.11	0.01	0.41	0.07	0.14	0.01	0.40	0.01	0.32	0.17	0.19	0.10	0.88	0.88	0.21	0.03	0.25	0.08	0.91	0.36	0.15	0.37	0.40	0.09	

Values shown above the diagonal are *D'* and values shown below are r^2.

TABLE III. Haplotypic Analysis of *PTPRZ1*

No.	SNP ID	Haplotypic global *P*-value window size 2	3	4
1	rs13437930			
		0.22		
2	rs2402593		0.38	
		0.25		0.57
3	rs1916885		0.46	
		0.64		0.60
4	rs3757548		0.66	
		0.84		0.77
5	rs1206477		0.74	
		0.54		0.90
6	rs6974265		0.77	
		0.64		0.50
7	rs1011692		0.37	
		0.15		0.45
8	rs1860721		0.31	
		0.21		0.26
9	rs4731044		0.34	
		0.58		0.40
10	rs6466808		0.44	
		0.41		0.35
11	rs1196482		0.28	
		0.21		0.24
12	rs10278079		0.19	
		0.18		0.26
13	rs1196511		0.26	
		0.16		0.17
14	rs3817483		0.21	
		0.13		0.41
15	rs10225212		0.29	
		0.21		0.34
16	rs1196471		0.23	
		0.84		0.44
17	rs2693657		0.96	
		0.92		0.44
18	rs1196474		0.43	
		0.26		0.41
19	rs17144005		0.33	
		0.27		0.26
20	rs1147502		0.27	
		0.51		0.43
21	rs12670616		0.73	
		0.58		0.07
22	rs1147497		0.62	
		0.62		0.64
23	rs1147492		0.62	
		0.57		0.39
24	rs1147491		0.32	
		0.22		0.48
25	rs1918031		0.45	
		0.47		0.54
26	rs1147489		0.39	
		0.50		0.36
27	rs1147488		0.37	
		0.34		
28	rs1206381			

Haplotypic global *P*-value was calculated using Unphased software.

specific than phenotypes) or symptoms are also thought to be important in this field [Gottesman and Gould, 2003; Craddock et al., 2006; Braff et al., 2007]. We did not take advantage of these analyses in order to test for a genetic association, but they may be useful in elucidating the potential role of *PTPRZ1* in schizophrenia. Because several studies have demonstrated that *Ptprz*-deficient mice suffer hippocampal dysfunction [Niisato et al., 2005; Tamura et al., 2006], additional endophenotypic approaches such as cognitive function assessment, brain imaging and other phenotypes that reveal *PTPRZ1* traits would further contribute to our understanding of schizophrenia.

In summary, the findings of the present study suggest that *PTPRZ1* is unlikely to be related to the development of schizophrenia in the Japanese population. Further replication studies incorporating supplemental populations should be performed for conclusive results.

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A genetic association study of the FXYD domain containing ion transport regulator 6 (*FXYD6*) gene, encoding phosphohippolin, in susceptibility to schizophrenia in a Japanese population

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ABSTRACT

The FXYD domain containing ion transport regulator 6 (*FXYD6*) gene is located within a region of chromosome 11 (11q23.3) that has been shown by a number of genome scans to be one of the most well-established linkages to schizophrenia. *FXYD6* encodes the protein phosphohippolin, which is primarily expressed in the brain. Phosphohippolin modulates the kinetic activity of Na,K-ATPase and has long-term physiological importance in maintaining cation homeostasis. A recent study reported that *FXYD6* was associated with schizophrenia in the United Kingdom samples. Applying the gene-based association concept, we carried out an association study regarding *FXYD6* and schizophrenia in a Japanese population, with a sample consisting of 2026 subjects (906 schizophrenics and 1120 controls). After linkage disequilibrium analysis, 23 single nucleotide polymorphisms (SNPs) were genotyped using 5′-exonuclease allelic discrimination assay. We found a significant association of two SNPs (rs11216573: genotypic *P* value: 0.022 and rs555577: genotypic *P* value: 0.026, allelic *P* value: 0.011, uncorrected). Nominal *P* values did not survive correction for multiple testing (rs11216573: genotypic *P* value: 0.47 and rs555577: genotypic *P* value: 0.55, allelic *P* value: 0.24, after SNPSpD correction). No association was observed between schizophrenia patients and controls in allelic, genotypic and haplotypic analyses. Our findings suggest that *FXYD6* is unlikely to be related to the development of schizophrenia in a Japanese population.

Schizophrenia is a severe and debilitating mental illness that affects approximately 1% of the population worldwide [21]. Although family, twin and adoption studies have demonstrated a high heritability (a heritability score of approximately 0.8), the molecular basis of the disease remains unclear [10,21]. The hypothesis that schizophrenia is a developmental disorder of the nervous system with a late onset of characteristic symptoms has been gaining acceptance over the past years, and the involvement of several candidate predisposition genes, such as neuregulin-1 (*NRG1*), disrupted-in-schizophrenia 1 (*DISC1*), and dysbindin (*DTNBP1*), in the development of schizophrenia has been reported [13,20]. Likewise, a hypothetical model based on deficient glutamatergic neurotransmission in the prefrontal cortex has been examined in the aetiology of schizophrenia [15]. In the central nervous system (CNS), most of the excitatory neurotransmission uses L-glutamate as the principal neurotransmitter. Glutamate transport depends on Na^+, K^+ transmembrane gradients generated by Na,K-ATPase [24].

The FXYD domain containing ion transport regulator 6 (*FXYD6*; located on 11q23.3) gene is a member of a family of seven FXYD genes. The chromosome 11q22-24 region has been shown to be one of the most well-established linkages to schizophrenia by a meta-analysis of 20 genome scans and other studies of genome scans (locus SCZD; MIM #181500) [12,14,16]. The FXYD proteins share homology for a single common transmembrane domain [25]. Each FXYD protein is expressed in a tissue-specific manner and functions by altering the kinetic activity of Na,K-ATPase.

Phosphohippolin, one of the FXYD proteins, modulates the kinetic activity of Na,K-ATPase and has long-term physiological importance in maintaining cation homeostasis [8]. *FXYD6* encodes

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the protein phosphohippolin, which is primarily expressed in the brain and kidney [28]. In rats, phosphohippolin has been found to be expressed in the neuronal fibers of the medial part of lateral habenula nucleus, thalamus, hypothalamus, stria terminalis, zona incerta, amygdaloid body, cingulum, olfactory bulb, hippocampus, cerebral cortex, and cerebellum [11]. Expression studies of the brain during development show the greatest amount of phosphohippolin in the postnatal 3-week-old rat brain, with a substantial capacity for phosphohippolin still existing in the adult brain [11]. This suggests that phosphohippolin may play an important role in neuronal excitability of the CNS during postnatal development, as well as that in the adult brain [11]. In humans, the expression of *FXYD6* is primarily in the brain, with the highest level of expression found in the fetal brain, prefrontal cortex, amygdala, occipital lobe, and hypothalamus according to the Novartis gene-expression-atlas database (GNF Sym-Atlas; Human GeneAtlas GNF1H, gcRMA; http://symatlas.gnf.org/SymAtlas/) and the GeneNote database (http://www.genecards.org/). The notable levels of expression occur in regions of the brain thought to be involved in schizophrenia, as identified by brain-imaging abnormalities [26]. In a postmortem brain study of schizophrenia and bipolar disorder, the expression of *FXYD6* in the dorsolateral prefrontal cortex (Brodmann area 46) was tended to be decreased compared with healthy subjects (the Stanley Brain Collection, http://www.stanleyresearch.org/brain/). *FXYD6* is, therefore, a positional and functional candidate gene for schizophrenia.

Recently, a case–control association study between two genes located on 11q23.3 (*FXYD2* and *FXYD6*) and schizophrenia was carried out, and *FXYD6* was demonstrated to be associated with schizophrenia in United Kingdom samples (rs3168238: $P = 0.009$; odds ratio 1.64, rs1815774: $P = 0.049$; odds ratio 1.21, rs4938445: $P = 0.010$; odds ratio 1.31, rs4938446: $P = 0.025$; odds ratio 1.26 and rs497768: $P = 0.023$; odds ratio 1.24) [5]. However, although *FXYD6* is considered one of the plausible candidate genes for schizophrenia, a replication of this positive association is required to maintain that *FXYD6* is a true susceptibility gene for schizophrenia.

It is widely accepted that there are differences in allele frequency or variations of the linkage disequilibrium (LD) structure (population dependence) among each ethnicity. Therefore, a gene-based approach, rather than a single nucleotide polymorphism (SNP)-based or haplotype-based approach, is currently recommended [17]. In such studies, it is important to include both the gene as well as the gene flanking regions when testing for any association, and it is also important to select genetic variants that adequately reflect the LD background by the standardized disequilibrium coefficient (D') and squared correlation coefficient (r^2) in the targeted population (e.g., tagging SNPs).

By applying the aforementioned concept, we carried out a gene-based association study between *FXYD6* and schizophrenia in a Japanese population to try to replicate previous findings.

The total sample used in this research, comprising 2026 subjects (906 schizophrenics and 1120 controls). The cohorts consisted of 906 patients with schizophrenia (513 males and 393 females, mean age ± S.D. = 42.3 ± 17.5 years) and 1120 healthy control subjects (559 males and 561 females, mean age ± S.D. = 38.6 ± 15.4 years). All subjects were unrelated to each other and were of Japanese ethnicity. The patients were diagnosed according to the DSM-IV-TR (Diagnostic and Statistical Manual of Mental Disorders, Fourth Edition, Text Revision) criteria for schizophrenia with the consensus reached among at least two experienced psychiatrists on the basis of unstructured interviews as well as a review of the subjects' medical records. All healthy control subjects were also psychiatrically screened on the basis of brief diagnostic unstructured interviews. Subjects who had current or past contact with psychiatric services were excluded. After describing the study to each subject, written informed consent was obtained. This study was approved by the Ethics Committee of the Nagoya University Graduate School of Medicine and Fujita Health University School of Medicine.

We used a gene-based approach to analyze genetic associations. This method implies the inclusion of both the gene region and gene flanking regions in the association study [17]. *FXYD6* contains eight exons spanning approximately 39,700 base pairs (bp) and no validated splicing isoform has been annotated thus far (RefSeq NCBI). We first consulted the HapMap database (http://www.hapmap.org/; release#22; phase2, April 2007, population: Japanese in Tokyo). All common SNPs (minor allele frequency (MAF) > 0.05) in the entire gene region, as well as the 5000 bp upstream 5′ flanking region and 5000 bp downstream 3′ flanking region were listed.

Then we defined 22 tagging SNPs (Table 1) with the criterion of an $r^2 > 0.8$ in 'pair-wise tagging only' mode using the 'Tagger' program, implemented by Haploview software version 4.0 (http://www.broad.mit.edu/mpg/haploview/index.php) [2,7]. In other words, if r^2 calculated from HapMap data was more than 0.8, only one of the two loci was selected for the association study. Significant SNPs (rs1815774, rs4938445 and rs497768) in the previous study [5] were selected by 'Tagger' (Table 1). rs4938446 was not selected because its r^2 between rs4938445 was 1.0, but we included rs4938446 in our analysis. rs3168238 was not included in our analysis, because it was not polymorphic in the Japanese population (dbSNP NCBI, HapMap database). Overall, 23 SNPs were examined.

Venous blood was drawn from the subjects and genomic DNA was extracted from whole blood according to standard procedures. Genotyping of all tagging SNPs was carried out using TaqMan 5′-exonuclease allelic discrimination assay (Applied Biosystems Japan Ltd., Tokyo, Japan). TaqMan probes and Universal PCR Master Mix were obtained from Applied Biosystems. Allelic-specific fluorescence was measured using the ABI PRISM 7900 Sequence Detector System (Applied Biosystems). Details regarding reagents or reaction conditions are available upon request.

Genotypes were tested for Hardy–Weinberg equilibrium using the chi-square goodness-of-fit test. Single-marker allelic and genotypic associations were evaluated using the chi-square test or Fisher's exact test. Genotypic association of SNPs that deviated from HWE was analyzed using Cochran–Armitage trend test for a multiplicative model of inheritance [1]. Analysis of LD between markers (r^2 and D') was performed using Haploview software. For haplotype-wise analysis, LD blocks were initially defined in accordance with Gabriel's criteria using Haploview software. Haplotypic analyses within LD blocks were performed with Unphased software version 2.403 [9], which performs log-likelihood ratio tests under a log-linear model for global P values. Rare haplotypes found in less than 5% of both case and control subjects were excluded from the association analysis to provide greater sensitivity and accuracy when the effect was seen in common haplotypes, and the expectation-maximization algorithm was then used. We also used in a 2- to 5-marker sliding window fashion analysis. The significance level for all statistical tests was $P < 0.05$. Power calculations were performed using the web-based genetic statistical package Genetic Power Calculator [19] (http://pngu.mgh.harvard.edu/~purcell/gpc/). Power was estimated under a multiplicative model of inheritance, assuming the disease prevalence to be 1% and the population susceptibility allele frequencies to be the values observed in control samples. The number of effective independent SNPs assayed was estimated by the spectral decomposition method of Nyholt using SNPSpD software. This software is able to reflect the correlation of markers (LD) on corrected P values to control for inflation of the type I error rate [18] (http://gump.qimr.edu.au/general/daleN/SNPSpD/).

Table 1
Association analysis of 23 SNPs with *FXYD6*

No.	SNP ID		M/m	N CON	N SCZ	CON M/M	CON M/m	CON m/m	SCZ M/M	SCZ M/m	SCZ m/m	MAF CON	MAF SCZ	HWE *P* value	Genotypic *P* value	[a,b,c]	[g]	Allelic *P* value	[d,g]	LD Block [d]	Haplotypic global *P* value	[e]	GRR [f]
1	rs11216573		G/A	1084	895	583	417	84	482	370	43	0.27	0.26	0.47	**0.022**	a	0.47	0.28		/			1.22
2	rs2282506		C/T	1092	892	958	131	3	762	124	6	0.06	0.08	0.74	0.19	b		0.09		/			1.40
3	rs520333		C/T	1079	893	607	396	76	469	349	75	0.25	0.28	0.33	0.21	a		0.07		I			1.22
4	rs564989		T/C	1078	892	469	485	124	414	395	83	0.34	0.31	1.00	0.20	a		0.09		I			1.21
5	rs10892185		G/C	1081	896	510	455	116	417	394	85	0.32	0.32	0.36	0.55	a		0.84		I	0.21		1.21
6	rs496371		C/T	1077	896	753	294	30	603	268	25	0.16	0.18	0.90	0.44	a		0.28		I			1.26
7	rs555577		G/A	1076	893	502	464	110	363	423	107	0.32	0.36	0.90	**0.026**	a	0.55	**0.011**	0.24	I			1.21
8	rs7121573	*	A/G	1091	892	630	396	65	512	307	73	0.24	0.25	0.84	0.14	a		0.35		II			1.23
9	rs1815774	**	C/G	1083	894	619	399	65	512	313	69	0.24	0.25	1.00	0.28	a		0.56		II			1.23
10	rs11216591		A/T	1078	894	587	413	78	479	354	61	0.26	0.27	0.69	0.82	a		0.87		II	0.69		1.22
11	rs11216594		A/G	1087	894	600	410	77	486	331	77	0.26	0.27	0.58	0.45	a		0.40		II			1.22
12	rs876797	*	C/A	1089	894	627	395	67	517	327	50	0.24	0.24	0.69	0.87	a		0.77		II			1.23
13	rs10790218	*	G/A	1088	894	341	521	226	283	434	177	0.45	0.44	0.32	0.87	a		0.68		III			1.20
14	rs4938445	**	G/A	1042	897	798	220	24	710	173	14	0.13	0.11	0.09	0.28	a		0.11		III	0.63		1.29
15	rs4938446	**	T/A	1080	895	822	238	20	705	175	15	0.13	0.12	0.64	0.37	a		0.18		III			1.29
16	rs7119090		G/C	1078	892	470	488	120	356	428	108	0.34	0.36	0.76	0.25	a		0.13		/			1.21
17	rs11216598		A/G	1077	890	464	489	124	414	376	100	0.34	0.32	0.84	0.30	a		0.22		/			1.21
18	rs631898		G/A	1085	896	627	397	61	513	321	62	0.24	0.25	0.94	0.49	a		0.50		/			1.23
19	rs11605223		C/G	1088	893	814	249	25	694	184	15	0.14	0.12	0.30	0.27	a		0.10		/			1.28
20	rs3809044		T/C	1093	896	942	143	8	773	119	4	0.07	0.07	0.42	0.76	b		0.82		IV			1.38
21	rs3809043		C/G	1094	895	872	201	21	716	170	9	0.11	0.11	**0.04**	0.55	c		0.54		IV	0.96		1.31
22	rs3809042		G/C	1092	896	692	353	47	555	310	31	0.21	0.21	0.87	0.40	a		0.82		IV			1.24
23	rs497768	**	G/C	1073	892	428	486	159	357	419	116	0.38	0.37	0.30	0.49	a		0.53		/			1.20

N, number; M, major allele; m, minor allele; CON, control; SCZ, schizophrenia; MAF, minor allele frequency; HWE, Hardy–Weinberg equilibrium; LD, linkage disequilibrium; GRR, genotype relative risk ($\alpha = 0.05$, $1 - \beta = 0.8$).
IDs with asterisk represent significant (**) or marginally significant (*) SNPs in Choudhury's report.
Genotypic *P*-value was calculated by the chi-square test (a), Fisher's exact test (b) and Cochran–Armitage trend test (c).
Allelic *P*-value was calculated and LD block (Gabriel's criteria) was defined using Haploview software (d).
Haplotypic global *P*-value was calculated using Unphased software (e).
GRR was calculated using Genetic power calculator (f).
Bold numbers represent significant *P*-values (uncorrected).
Corrected *P*-value was calculated by Nyholt's method (g).
Effective number of independent marker loci: 21.38
Experiment-wide significance threshold required to keep Type I error rate at 5%: 0.0023.

Table 2
Linkage disequilibrium analysis of *FXYD6*

No.		1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23
1	rs11216573		0.53	0.66	0.69	0.56	0.67	0.76	0.42	0.43	0.45	0.46	0.43	0.35	0.00	0.01	0.51	0.19	0.08	0.02	0.01	0.07	0.29	0.25
2	rs2282506	0.01		0.96	0.76	1.00	0.64	0.90	0.51	0.55	0.28	0.56	0.21	0.46	0.85	0.78	0.07	0.46	0.12	0.02	1.00	0.04	0.12	0.12
3	rs520333	0.05	0.18		0.96	0.99	0.99	0.97	0.53	0.54	0.21	0.52	0.24	0.35	0.48	0.55	0.12	0.37	0.10	0.45	0.06	0.42	0.01	0.14
4	rs564989	0.34	0.02	0.16		0.97	0.98	0.99	0.35	0.35	0.60	0.33	0.56	0.35	0.02	0.01	0.53	0.24	0.02	0.11	0.09	0.03	0.24	0.17
5	rs10892185	0.05	0.03	0.15	0.22		1.00	0.99	0.87	0.88	0.45	0.79	0.47	0.49	0.11	0.12	0.31	0.14	0.02	0.10	0.05	0.14	0.44	0.18
6	rs496371	0.03	0.01	0.56	0.10	0.09		1.00	0.45	0.48	0.13	0.49	0.25	0.27	0.16	0.29	0.34	0.36	0.25	0.56	0.10	0.53	0.23	0.38
7	rs555577	0.10	0.12	0.68	0.23	0.21	0.42		0.56	0.56	0.28	0.53	0.29	0.21	0.32	0.39	0.01	0.41	0.05	0.10	0.03	0.21	0.14	0.18
8	rs7121573*	0.02	0.06	0.27	0.02	0.11	0.13	0.21		0.98	0.97	0.97	0.97	0.88	0.60	0.60	0.70	0.23	0.16	0.14	0.17	0.19	0.04	0.00
9	rs1815774**	0.02	0.06	0.28	0.02	0.12	0.14	0.22	0.94		1.00	1.00	1.00	0.91	0.63	0.63	0.71	0.24	0.17	0.15	0.18	0.16	0.02	0.01
10	rs11216591	0.03	0.00	0.01	0.07	0.16	0.00	0.01	0.11	0.12		0.89	0.97	0.92	0.97	1.00	0.93	0.31	0.07	0.02	0.16	0.20	0.48	0.43
11	rs11216594	0.03	0.06	0.27	0.02	0.10	0.13	0.21	0.87	0.93	0.10		1.00	0.90	0.65	0.66	0.62	0.25	0.18	0.18	0.16	0.15	0.05	0.01
12	rs876797*	0.02	0.00	0.01	0.05	0.15	0.00	0.01	0.10	0.10	0.85	0.11		0.98	0.82	0.85	0.93	0.32	0.08	0.05	0.14	0.19	0.66	0.50
13	rs10790218*	0.04	0.01	0.03	0.05	0.14	0.01	0.02	0.20	0.22	0.38	0.23	0.38		0.94	0.94	0.79	0.28	0.08	0.03	0.03	0.03	0.43	0.22
14	rs4938445**	0.00	0.01	0.01	0.00	0.00	0.00	0.01	0.02	0.02	0.05	0.02	0.03	0.16	0.91	0.97	0.94	0.29	0.11	0.04	0.60	0.10	0.46	0.02
15	rs4938446**	0.00	0.01	0.02	0.00	0.00	0.00	0.01	0.02	0.02	0.05	0.02	0.04	0.16	0.91		0.98	0.33	0.09	0.04	0.57	0.11	0.51	0.00
16	rs7119090	0.05	0.00	0.00	0.07	0.09	0.01	0.00	0.08	0.08	0.60	0.07	0.55	0.39	0.07	0.07		0.33	0.03	0.20	0.07	0.40	0.46	0.40
17	rs11216598	0.03	0.01	0.03	0.06	0.01	0.01	0.04	0.01	0.01	0.02	0.01	0.02	0.03	0.01	0.01	0.03		0.31	0.03	0.02	0.17	0.29	0.18
18	rs631898	0.01	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.01	0.00	0.01	0.00	0.00	0.02		0.28	0.37	0.01	0.04	0.05
19	rs 11605223	0.00	0.00	0.01	0.00	0.00	0.01	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.04		0.77	0.72	0.81	0.48
20	rs3809044	0.00	0.01	0.00	0.00	0.00	0.00	0.00	0.00	0.01	0.01	0.01	0.01	0.00	0.00	0.00	0.00	0.00	0.00	0.01		1.00	1.00	0.84
21	rs3809043	0.00	0.00	0.01	0.00	0.01	0.01	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.01	0.01	0.01	0.01	0.00	0.41	0.01		0.93	0.58
22	rs3809042	0.06	0.00	0.00	0.03	0.02	0.00	0.00	0.00	0.00	0.02	0.00	0.04	0.04	0.01	0.01	0.03	0.04	0.00	0.03	0.02	0.03		0.91
23	rs497768**	0.04	0.00	0.00	0.03	0.01	0.02	0.01	0.00	0.00	0.04	0.00	0.05	0.02	0.00	0.00	0.05	0.03	0.00	0.06	0.03	0.07	0.36	

Values shown above the diagonal are D' and values shown below are r^2.

Table 3
Haplotypic analysis of *FXYD6*

No.	SNPID	Haplotypic global *P* value Window size			
		2	3	4	5
1	rs11216573				
		0.27			
2	rs2282506		0.36		
		0.54		0.32	
3	rs520333		0.35		0.46
		0.66		0.49	
4	rs564989		0.63		0.56
		0.49		0.67	
5	rs10892185		0.53		0.21
		0.90		0.16	
6	rs496371		0.11		0.47
		0.09		0.40	
7	rs555577		0.36		0.26
		0.70		0.26	
8	rs7121573*		0.56		0.15
		0.63		0.39	
9	rs1815774**		0.66		0.26
		0.65		0.43	
10	rs11216591		0.44		0.66
		0.58		0.66	
11	rs11216594		0.69		0.81
		0.55		0.86	
12	rs876797		0.75		0.79
		0.59		0.78	
13	rs10790218*		0.57		0.72
		0.32		0.56	
14	rs4938445**		0.61		0.50
		0.88		0.64	
15	rs4938446**		0.89		0.60
		0.80		0.27	
16	rs7119090		0.58		0.62
		0.28		0.89	
17	rs11216598		0.49		0.95
		0.97		0.69	
18	rs631898		0.97		0.77
		0.69		0.88	
19	rs 11605223		0.66		0.94
		0.25		0.86	
20	rs3809044		0.26		0.77
		0.98		0.93	
21	rs3809043		0.96		0.73
		0.78		0.67	
22	rs3809042		0.59		
		0.76			
23	rs497768**				

Haplotypic global *P* value was calculated using Unphased software.
IDs with asterisk represent significant (**) or marginally significant (*) SNPs in Choudhury's report.

The genotype and allele frequency of each SNP in schizophrenic patients and healthy control subjects are summarized in Table 1. The observed genotype frequency of rs3809043 deviated from HWE. The LD relationships between markers are shown in Table 2. The LD patterns observed in controls were nearly identical to those of the JPT HapMap samples, but obviously different from those of the CEU HapMap samples. The haplotypic analysis is provided in Tables 1 and 3. We found a significant association of two tagging SNPs (rs11216573; genotypic *P* value: 0.022 and rs555577; genotypic *P* value: 0.026, allelic *P* value: 0.011) before correction for multiple testing. To correct for multiple testing, we estimated the effective number of independent SNPs using the method of Nyholt [18]. The number of independent SNPs was estimated at 21.38. Thus the gene-wide corrected *P* value for significance was 0.002338. Nominal *P* values did not survive correction for multiple testing. No association was observed between schizophrenia patients and controls in allelic and genotypic analyses after correction for multiple testing (Table 1). More than 80% power in detecting an associa-

tion with schizophrenia was obtained when the genotype relative risk (GRR) was set at 1.20–1.40 under a multiplicative model of inheritance.

The "common disease-common variant" hypothesis postulates that LD should be detected by the haplotype association test if the risk haplotype is linked to causal variants for disease [4]. Regarding the Japanese population, therefore, the data presented in this article do not provide sufficient evidence for the involvement of *FXYD6* in conferring susceptibility for schizophrenia.

In this study, we found a significant association of two tagging SNPs (rs11216573 and rs555577) before correction for multiple testing. It is important to control for inflation of type I errors due to multiple testing. Bonferroni correction is typically used for solving multiple testing problems; however, because markers are not independent due to the existence of LD, Bonferroni correction is thought to be too conservative. We thus performed multiple testing correction by the spectral decomposition method of Nyholt using SNPSpD software. Nominal *P* values did not survive correction for multiple testing (rs11216573; genotypic *P* value: 0.47 and rs555577; genotypic *P* value: 0.55, allelic *P* value: 0.24, corrected by the spectral decomposition method of Nyholt). The significant association in our results was thought to be a false-positive finding due to multiple testing. Over time, we could not replicate a previous report [5], which revealed a significant association between *FXYD6* and schizophrenia in a Caucasian population. The discrepancy between Japanese and Caucasians may derive from ethnic differences in the etiology of schizophrenia. Although the sample size used here is smaller than the sample from the original study, we obtained more than 80% power in detecting any possible association; thus the possibility of a type II error is less likely. The GRR value predefined in our power analysis was appropriate when compared with Choudhury's report (odds ratio: 1.21–1.64) and other promising candidate genes for schizophrenia [22,23]. We also performed haplotypic analysis excluding rs4938446, because this SNP was not selected by Tagger. However, no significant results were obtained (data not shown).

Two additional limitations need to be addressed to discuss the present results. First, age-unmatched and male-to-female ratio unmatched cohorts were examined in the present study. The mean age of the controls was younger than that of patients. This means that a number of these younger controls, though likely not more than 12 subjects given a lifetime morbidity risk of 1%, may later develop schizophrenia. In order to address this issue, we also included an explorative analysis of gender effect. However, no associations were found in analysis subdivided by gender (supplementary Table 1).

Second, the definition of phenotypes is vital for a genetic association study. Therefore, endophenotypes (being more specific than phenotypes) or symptoms are also thought to be important in this field [3,6]. We did not take advantage of these analyses to test for a genetic association; however, those analyses might be useful in elucidating the potential role of *FXYD6* in schizophrenia. Additional endophenotypic approaches, such as cognitive function assessment, brain imaging, and other phenotypes that reveal *FXYD6* traits would further contribute to our understanding of schizophrenia. In this regard, a recent study reported that *DAOA/G30* influences susceptibility to the symptomatology of psychiatric disorders including schizophrenia and bipolar disorder, but not to the diagnosis itself [27]. The phenotypic definition should be considered in future genetic association studies.

In summary, the findings of this present study suggest that *FXYD6* is unlikely to be related to the development of schizophrenia in a Japanese population. Further replication studies incorporating supplemental populations are required for conclusive results.

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Competing interests: None.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.neulet.2008.04.010.

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Identification of *YWHAE*, a gene encoding 14-3-3epsilon, as a possible susceptibility gene for schizophrenia

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Schizophrenia is a complex mental disorder with a fairly high degree of heritability. Although the causes of schizophrenia remain unclear, it is now widely accepted that it is a neurodevelopmental and neurodegenerative disorder involving disconnectivity and disorder of the synapses. Disrupted-in-schizophrenia 1 (*DISC1*) is a promising candidate susceptibility gene involved in neurodevelopment, including maturation of the cerebral cortex. To identify other susceptibility genes for schizophrenia, we screened for *DISC1*-interacting molecules [NudE-like (*NUDEL*), Lissencephaly-1 (*LIS1*), 14-3-3epsilon (*YWHAE*), growth factor receptor bound protein 2 (*GRB2*) and Kinesin family 5A of Kinesen1 (*KIF5A*)], assessing a total of 25 tagging single-nucleotide polymorphisms (SNPs) in a Japanese population. We identified a *YWHAE* SNP (*rs28365859*) that showed a highly significant difference between case and control samples, with higher minor allele frequencies in controls ($P_{allele} = 1.01 \times 10^{-5}$ and $P_{genotype} = 4.08 \times 10^{-5}$ in 1429 cases and 1728 controls). Both messenger RNA transcription and protein expression of 14-3-3epsilon were also increased in the lymphocytes of healthy control subjects harboring heterozygous and homozygous minor alleles compared with homozygous major allele subjects. To further investigate a potential role for *YWHAE* in schizophrenia, we studied *Ywhae*$^{+/-}$ mice in which the level of 14-3-3epsilon protein is reduced to 50% of that in wild-type littermates. These mice displayed weak defects in working memory in the eight-arm radial maze and moderately enhanced anxiety-like behavior in the elevated plus-maze. Our results suggest that *YWHAE* is a possible susceptibility gene that functions protectively in schizophrenia.

INTRODUCTION

Recent neuroimaging studies show that structural brain abnormalities are an established feature of schizophrenia and are characterized by decreased total gray matter volume (1,2). These morphological correlates of schizophrenia range from a reduction in brain size to localized alterations in the morphology and molecular composition of specific neuronal, synaptic and glial populations in specific brain areas such as the hippocampus, dorsolateral prefrontal cortex and dorsal thalamus.

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These findings have fostered the current view of schizophrenia as a disorder of connectivity (3,4) and of the synapse (5). Although the mechanism underlying the neurodevelopmental/neurodegenerative process is still unclear, a way forward is provided by the recent identification of several putative susceptibility genes, such as *Neuregulin 1* (6), *Dysbindin* (7), *G72* (8), Catechol-*O*-methyltransferase (*COMT*) (9–11) and others (12,13). For none of these genes, however, has a causative allele or the mechanism by which it predisposes to schizophrenia been identified.

Disrupted-in-schizophrenia 1 (*DISC1*) was first described as a strong candidate gene in a large Scottish family in which a balanced chromosomal translocation segregates with schizophrenia and other psychiatric disorders (12,14,15). The translocation mutation may result in loss of DISC1 function via haploinsufficiency or dominant-negative effects of a predicted mutant DISC1 truncated protein product. *DISC1* has been implicated in neurodevelopment, including maturation of the cerebral cortex (16).

DISC1 interacts with several proteins, including NudE-like (NUDEL) (17–19), lissencephaly-1 (LIS1, also called PAFAH1B1) (20), fasciculation and elongation protein zeta 1 (FEZ1) (21) and phosphodiesterase 4B (PDE4B) (16). Recently, we identified several novel DISC1-interacting molecules, including 14-3-3epsilon, Kinesin family 5A of Kinesen1 (KIF5A) and Growth factor receptor bound protein 2 (Grb2) by affinity column chromatography (22,23). Furthermore, we confirmed that DISC1 regulates the localization of the NUDEL/LIS1/14-3-3epsilon complex or Grb2 into axons as a cargo receptor (22,23) and it also regulates Neurotrophin-induced axon elongation by Grb2 (23).

In this study, we screened for the genetic association of DISC1-interacting molecules—*NUDEL* (17p13.1, OMIM: *607538), *LIS1* (17p13.3, OMIM: #607432), 14-3-3epsilon (17p13.3, OMIM: *605066), *Grb2* (17p24-q23, OMIM: *108355) and *KIF5A* (12q13, OMIM: *602821)—with schizophrenia, and identified the gene encoding 14-3-3epsilon (*YWHAE*) as a possible susceptibility gene. Our results show that a SNP of *YWHAE*, which influence the expression of 14-3-3epsilon RNA and protein, is associated with schizophrenia and seems to work protectively. We also investigated the behavioral phenotype of mice with ~50% reduction in 14-3-3epsilon protein expression and found that these mice displayed weak phenotypes consistent with some aspects of human schizophrenia.

RESULTS

Screening analysis of *DISC1*-related genes and identification of *YWHAE* as a possible susceptibility gene for schizophrenia

To investigate whether novel DISC1-interacting molecules such as NUDEL, LIS1, YWAHE, GRB2 and KIF5A are associated with schizophrenia, we performed genetic association analyses using a Japanese population.

We failed to develop the genotyping of three SNPs in *LIS1* (*rs8082331*, *rs12938775* and *rs4790348*) and one SNP in *GRB2* (*rs16967795*), therefore a total of 25 SNPs were assessed in this analysis.

Though genotype distributions of two SNPs significantly deviated from Hardy–Weinberg Equilibrium (HWE, $P_{HWE} = .0143$: *rs4789172* in case sample, and $P_{HWE} = .0171$: *rs11172247* in control sample), those of the other markers were in HWE. Six tagging SNPs in *YWHAE* were significantly associated with schizophrenia and also *YWHAE* showed gene-wide significance (permutation $P = 0.0021$), whereas we found no association of tagging SNPs in *NUDEL*, *LIS1*, *GRB2* or *KIF5A* (Table 1).

Since six tagging SNPs in *YWHAE* located in the intron region, we performed denaturing high-performance liquid chromatography (dHPLC) analysis in 5′ flanking regions and entire exon regions of *YWHAE* to identify the possible causal polymorphism, and detected two SNPs: one in the 5′ flanking region (−261 bp from the initial exon: *rs28365859*) and the other one in the 3′-UTR (*rs9393*). Since the 5′ flanking region SNP might have a functional effect due to its position, we focused on this SNP in the following analysis [linkage disequilibrium (LD) structure of first-set samples in *YWHAE* can be seen in Fig. 1).

First, to examine the association of this SNP, we expanded the sample size (1065 cases and 1386 controls in a second set of confirmation samples, for a total of 1429 cases and 1728 controls including the first set of screening samples, call rates were 100%), and significant association was obtained ($P_{allele} = 1.01 \times 10^{-5}$ and $P_{genotype} = 4.08 \times 10^{-5}$). Furthermore, the significance could be detected in either set independently (Table 2). The commonly observed feature of these analyses was that the minor allele frequencies (MAFs) of this SNP were higher in controls than in schizophrenia patients. There was no discrepancy out of 380 randomly selected samples (190 cases and 190 controls) genotyped in duplicate and by another method (TaqMan Assay: C12125119) for this marker, suggesting it is unlikely that genotyping error had occurred.

Functional analysis of the promoter SNP in *YWHAE*: *in vitro* and *in vivo* expression assays

We first investigated the influence of *rs28365859* on *YWHAE* expression by dual-luciferase assay, although there is no evidence that the region where this SNP is located on is evolutionally conserved and that any regions in *YWHAE* are match as a core promoter by in silico promoter detection software. As shown in Fig. 2, a trend for significance in a promoterless vector and significance in a promoter vector were obtained in the different cell lines. The constructs containing a minor allele (C allele) showed higher expression in the promoter vector, suggesting that the C allele plays a possible enhancer role in these cell lines.

Next, to examine the role of this SNP in peripheral blood of healthy control subjects, real-time RT–PCR and western blot analysis were performed. Similar to the luciferase assays, heterozygous and homozygous minor allele (G/C and C/C genotype) subjects showed higher expression levels of 14-3-3epsilon than did homozygous major allele (G/G genotype) subjects (one-way analysis of variance, ANOVA, $P = 0.0251$ and 0.0014 in real-time RT–PCR and western blot analysis, respectively). Experimental analysis were performed to examine the differences under an additive model (G/G

Table 1. Screening analysis of DISC1-related genes

Gene	SNPs		Position[a]	Missing rate (%)	MAF Cases	Controls	*P*-value Allele	Genotype
NUDEL	rs3744652	C>T	8280008	0.3	33.0	35.9	0.250	0.274
	rs8064655	C>T	8301185	0	33.2	36.3	0.228	0.246
LIS1	rs1266474	A>G	2481460	0.4	9.72	12.4	0.110	0.0876
	rs4790356	G>A	2532979	0	10.6	11.7	0.528	0.730
	rs7212450	C>G	2538690	0	42.3	41.7	0.821	0.907
YWHAE	rs34041110	C>T	1193642	0	48.9	42.5	**0.0166**	**0.00563**
	rs9393	A>G	1195142	0	27.3	27.9	0.805	0.868
	rs8064578	C>T	1201625	0	48.5	43.4	0.0562	0.117
	rs7224258	G>C	1202252	2.1	15.0	20.3	**0.0102**	**0.0342**
	rs3752826	G>T	1211814	0	48.6	42.1	**0.0139**	**0.0175**
	rs7214541	T>C	1220072	0	44.6	49.4	0.0725	0.107
	rs11655548	A>G	1230748	2.3	29.3	38.3	**0.000418**	**0.00162**
	rs2131431	A>C	1241645	0.3	13.2	18.5	**0.00598**	**0.0176**
	rs1873827	A>G	1247690	0	42.4	49.6	**0.00732**	**0.0136**
	rs12452627	C>T	1249222	0	17.7	19.6	0.367	0.662
GRB2	rs7219	T>C	70826963	0	9.07	6.85	0.125	0.239
	rs8079197	C>G	70828274	0.6	8.45	6.60	0.190	0.308
	rs4789172	C>T	70853307	0.6	24.9[c]	26.1	0.617	0.659
	rs2053156	T>G	70890035	0	6.04	4.53	0.206	0.344
	rs930296	G>A	70915763	0	5.91	4.66	0.298	0.432
KIF5A	rs11172247	C>G	56232777	0	39.4	38.3[b]	0.676	0.609
	rs11172254	G>A	56255005	0.3	19.5	21.2	0.422	0.679
	rs775250	C>A	56263307	0	20.8	21.7	0.672	0.690
	rs775251	C>T	56265007	0.4	27.7	32.2	0.0713	0.129
	rs1678536	C>G	56265457	0.1	47.9	47.4	0.833	0.644

YWHAE showed gene-wide significance (permutation $P = 0.0021$).
Bold numbers represent significant *P*-values (<0.05).
[a]Based on HapMap database release#21.
[b]deviated from Hardy–Weinberg equilibrium.
MAF, minor allele frequency.

versus G/C+C/C), again significant associations were obtained.

Furthermore, haplotype trend regression test was applied to check the effects of haplotypes of *rs28365859* and other four SNPs in intron 1 (*rs11655548*, *rs2131431*, *rs1873827* and *rs12452627*), which might also be in an enhancer region. This showed significant association in either analysis ($P = 0.0282$ and 0.0186 in real-time RT–PCR and western blot analysis, respectively), however, each SNP in intron 1 was not correlated with the expression level (data not shown).

Effect of reduction of 14-3-3epsilon protein on the cognitive functions of mice

14-3-3 proteins are highly conserved across species, from bacteria to humans, and bind to phosphoserine/phosphothreonine motifs in a sequence-specific manner (24–28). Previously we reported that 14-3-3epsilon binds to CDK5/p35-phosphorylated NUDEL and maintains NUDEL phosphorylation. To examine the protective effect of 14-3-3epsilon on schizophrenia using mice, we should investigate whether overexpression of 14-3-3epsilon results in resistance for the onset of schizophrenic symptoms. However, an assay system to evaluate the effect of a gene on the onset of schizophrenia in mice has not yet been developed. Thus, in support of a role for *YWHAE* in schizophrenia, we investigated *Ywhae* knockout mice. Null mice of *Ywhae* gene ($Ywhae^{-/-}$) show a severe cell migration defect in both the cortex and the hippocampus, whereas $Ywhae^{+/-}$ mice, in which the expression level of 14-3-3epsilon protein is reduced to ~50% compared with their wild-type littermates, show a milder migration defect (29). Because most $Ywhae^{-/-}$ mice die at birth as previously reported (29), $Ywhae^{+/-}$ mice and their wild-type littermates were analyzed by a comprehensive behavioral test battery to investigate whether the reduction in 14-3-3epsilon protein affects behavior (30,31). $Ywhae^{+/-}$ mice appeared normal, healthy and fertile (Table 3).

To examine whether reduction in 14-3-3epsilon was associated with cognitive deficits, we analyzed $Ywhae^{+/-}$ mice and their wild-type littermates in working memory and reference memory tasks (Table 3). To assess working memory of $Ywhae^{+/-}$ mice, we used a spatial working memory version of the 8-arm radial maze task (32,33). The mice were trained for 26 trials. During training, both control and mutant mice improved their performance and no significant difference was observed ($P = 0.3325$) (Fig. 3A). The number of revisiting errors of $Ywhae^{+/-}$ mice was significantly more than their wild-type littermates during trials with a delay of 300 s ($P = 0.0229$) (Fig. 3C). The number of different arms chosen during the first eight choices, which is considered a measure of working memory that is relatively independent of locomotor activity levels and the total number of choices, was not significantly affected by the deficit of 14-3-3epsilon protein during training and trials with 30, 120 and 300 s of delay ($P = 0.3325$, 0.8972, 0.6476 and 0.5077, respectively) (Fig. 3B and D). These results

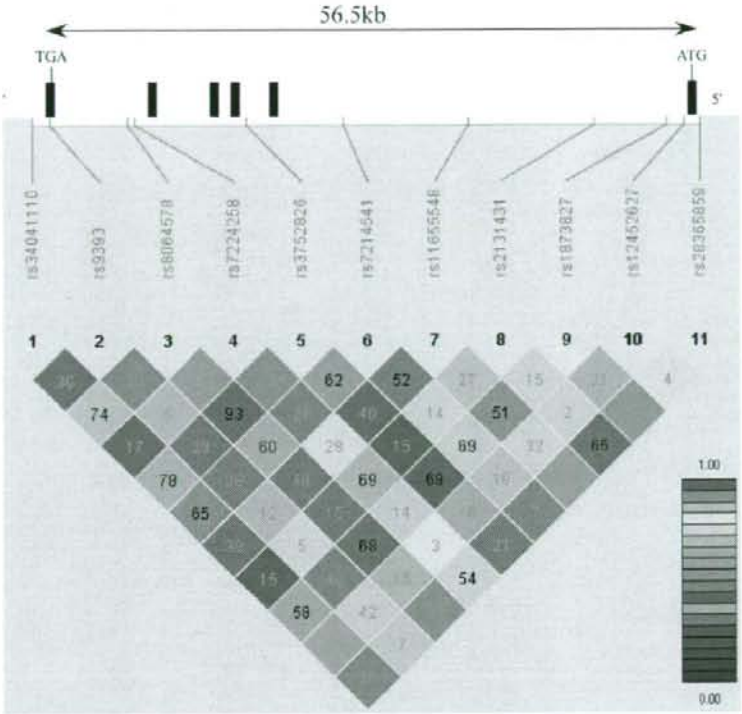

Figure 1. Tagging SNPs and LD evaluation of *YWHAE* for first-set screening samples. rs28365859 was included. Vertical bars represent exons. Numbers in boxes represent r^2 values, which should be expressed as decimals. r^2 values of 1.0 are not shown. Color scheme was based on GOLD format. Additional information is provided at the Haploview website.

Table 2. Association analysis of promoter SNP in YWHAE (rs28365859)

Samples[a]	Phenotype	*n*	Genotype G/G	G/C	C/C	MAF (%)	*P*-values HWE[b]	Allele	Genotype
Combined	Cases	1429	921	457	51	19.6	0.537	1.01×10^{-5}	4.08×10^{-5}
	Controls	1728	1000	620	108	24.2	0.366		
First-set	Cases	364	245	106	13	18.1	0.715	0.00108	0.00545
	Controls	342	192	127	23	25.3	0.748		
Second-set	Cases	1065	676	351	38	20.0	0.359	0.00123	0.00280
	Controls	1386	808	493	85	23.9	0.399		

First-set samples were identical to those used in screening analysis.
Second-set samples were independent set of samples to increase the sample size.
[a]Combined samples = first-set + second-set samples.
HWE, Hardy–Weinberg equilibrium.

suggest that *Ywhae*$^{+/-}$ mice show weak defects in working memory.

Next, we analyzed reference memory of *Ywhae*$^{+/-}$ mice, using the left–right discrimination test version of the T-maze. *Ywhae*$^{+/-}$ mice and their wild-type littermates were trained for 6 trials; then the correct side was reversed. The next 6 trials were performed under the reversal-learning condition. No significant difference was observed in the percentage of correct choices at the sixth trial (*Ywhae*$^{+/+}$, 80.647%; *Ywhae*$^{+/-}$, 77.157%; $P = 0.7516$), and no significant difference was observed under the reversal-learning condition ($P = 0.4567$) (Table 3). These results suggest that a decrease in the 14-3-3epsilon protein results in weak defects, specifically in spatial working memory.