(Conrad and Scheibel, 1987; Weinberger, 1987; Murray, 1994; Waddington et al., 1998). A recent study reported that SEMA3A was increased in the cerebellum in the postmortem brains of schizophrenia patients (Eastwood et al., 2003). More recently, a genome-wide association study using 25494 single nucleotide polymorphisms (SNPs) pointed out that an intronic SNP of the *PLXNA2* gene were most consistently associated with schizophrenia in a European American population (Mah et al., 2006). These findings suggested the possibility that genetic variations of the *PLXNA2* gene confer susceptibility to schizophrenia through its effects on neurodevelopment and synaptic plasticity. To our knowledge, however, the association between the *PLXNA2* gene and schizophrenia has not yet been supported or refuted by any other study. The present study was thus aimed to examine whether there is such an association in a Japanese sample. In addition, we examined 3 additional non-synonymous polymorphisms of the *PLXNA2* gene (Arg5Gln, GLn57Arg, and Ala267Thr) for association with schizophrenia.

2. Materials and methods

2.1. Subjects

Subjects were 336 patients with schizophrenia (204 males, mean age of 44.2 years [SD 13.7]) and 304 healthy controls (99 males, mean age of 38.8 years [SD 13.4]). All subjects were biologically unrelated Japanese and recruited from the same geographical area (Western part of Tokyo Metropolitan). Consensus diagnosis by at least two psychiatrists was made for each patient according to the Diagnostic and Statistical Manual of Mental Disorders, 4th edition (DSM-IV) criteria (American Psychiatric Association, 1994) on the basis of unstructured interviews and information from medical records. The controls were healthy volunteers recruited from hospital staffs and their associates. Control individuals were interviewed and those who had a current or past history of psychiatric treatment were not enrolled in the study. The study protocol was

Table 1
Genotype and allelic distribution of the *PLXNA2* SNPs in Japanese patients with schizophrenia and controls

dbSNP ID	Position[a]	Inter-SNP distance (bp)	Group	*N*	Genotype distribution (frequency)			Allele distribution (frequency)		Odds ratio (95% CI)	Chi-square test[b]		
											HWE (df=1)	GF (df=2)	AF (df=1)
rs2782948	206457877				CC	CT	TT	C	T				
	Exon 2	–	Schizophrenia	334	89 (0.27)	160 (0.48)	85 (0.25)	338 (0.51)	330 (0.49)	1.15	χ^2=0.58, P=0.45	P=0.31	P=0.23
	Arg5Gln		Control	303	65 (0.21)	156 (0.51)	82 (0.27)	286 (0.47)	320 (0.53)	(0.92–1.43)	χ^2=0.33, P=0.57	χ^2=2.34	χ^2=1.47
rs11119014	206457721				AA	AG	GG	A	G				
	Exon 2	156	Schizophrenia	334	265 (0.79)	61 (0.18)	8 (0.02)	591 (0.88)	77 (0.12)	0.82	χ^2=3.65, P=0.056	P=0.37	P=0.28
	Gln57Arg		Control	301	246 (0.82)	52 (0.17)	3 (0.01)	544 (0.90)	58 (0.10)	(0.57–1.17)	χ^2=0.02, P=0.90	χ^2=1.99	χ^2=1.19
rs3748735	206457092				CC	CT	TT	C	T				
	Exon 2	629	Schizophrenia	334	249 (0.75)	78 (0.23)	7 (0.02)	579 (0.86)	92 (0.14)	1.06	χ^2=0.09, P=0.76	P=0.31	P=0.77
	Ala267Thr		Control	303	228 (0.75)	63 (0.21)	12 (0.04)	519 (0.86)	87 (0.14)	(0.77–1.45)	χ^2=7.23, P=0.0072	χ^2=2.33	χ^2=0.09
rs2498028	206321936				GG	GA	AA	G	A				
	Intron11	135156	Schizophrenia	335	147 (0.44)	147 (0.44)	41 (0.12)	441 (0.66)	229 (0.34)	1.00	χ^2=0.21, P=0.65	P=0.65	P=0.99
	–		Control	303	128 (0.42)	143 (0.47)	32 (0.11)	399 (0.66)	207 (0.34)	(0.79–1.26)	χ^2=0.73, P=0.39	χ^2=0.87	χ^2=0.00
rs1327175	206313757				GG	GC	CC	G	C				
	Intron12	8179	Schizophrenia	334	266 (0.80)	63 (0.19)	5 (0.01)	595 (0.89)	73 (0.11)	0.92	χ^2=0.32, P=0.57	P=0.83	P=0.63
	–		Control	302	244 (0.81)	55 (0.18)	3 (0.01)	543 (0.90)	61 (0.10)	(0.64–1.31)	χ^2=0.00, P=0.96	χ^2=0.38	χ^2=0.23
rs752016	206304300				AA	AG	GG	A	G				
	Intron12	9457	Schizophrenia	334	106 (0.32)	153 (0.46)	75 (0.22)	365 (0.55)	303 (0.45)	0.96	χ^2=1.92, P=0.17	P=0.56	P=0.68
	–		Control	303	94 (0.31)	150 (0.50)	59 (0.19)	338 (0.56)	268 (0.44)	(0.77–1.19)	χ^2=0.00, P=0.95	χ^2=1.15	χ^2=0.17
rs841865	206292532				GG	GA	AA	G	A				
	Intron14	11768	Schizophrenia	335	171 (0.51)	132 (0.39)	32 (0.10)	474 (0.71)	196 (0.29)	1.11	χ^2=0.77, P=0.38	P=0.35	P=0.39
			Control	302	139 (0.46)	136 (0.45)	27 (0.09)	414 (0.69)	190 (0.31)	(0.87–1.41)	χ^2=0.59, P=0.44	χ^2=2.08	χ^2=0.73

[a] Chromosome position was referred to dbSNP database.
[b] Without correction.

Table 2
Pair-wise linkage disequilibrium and association with schizophrenia of the 7 SNPs and haplotypes in *PLXNA2*

dbSNP ID	Inter-SNP distance (bp)	LD		Haprotype P^a					
		D'	r^2	2 Locus	3 Locus	4 Locus	5 Locus	6 Locus	7 Locus
rs2782948	156	1.00	0.13	0.44					
rs11119014					0.48				
	629	1.00	0.02	0.49		0.67			
rs3748735					0.58		0.32		
	135156	0.30	0.03	0.70		0.53		0.11	
rs2498028					0.78		0.19		0.13
	8179	1.00	0.06	0.90		0.63		0.20	
rs1327175					0.73		0.38		
	9457	0.90	0.12	0.59		0.13			
rs752016					0.64				
	11768	0.88	0.42	0.53					
rs841865									

[a] Global P-value (without correction).

approved by the ethics committee at the National Center of Neurology and Psychiatry, Japan. After description of the study, written informed consent was obtained from every subject.

2.2. SNP selection

In the recent genome-wide association study for schizophrenia (Mah et al., 2006), the most consistent effect was observed for rs752016, a SNP within intron 12 of *PLXNA2*. For the initial discovery sample, the minor allele frequency was 0.22 in the control group and 0.16 in the case group (OR=1.49; P=0.006). To fine map the region of association, they analyzed additional 67 SNPs in a 100-kb window surrounding the marker SNP (rs752016) in the discovery case and control pools. Based on the association fine-mapping and genotyping results obtained in the case-control studies, 4 SNPs that best represented the region (rs752016, rs841865, rs1327175 and rs2498028) were genotyped in their collection. All the 4 SNPs showed a significant association with schizophrenia in their European American sample. Therefore, we chose these SNPs for replication study in our Japanese sample.

Since genetic variations that result in an amino acid change are most likely to alter functions, we searched for non-synonymous polymorphisms of *PLXNA2 in silico* based on the NCBI dbSNP database and found 3 well-validated SNPs with a heterozygosity value of >0.10. The selected ones are described in Table 1. They were rs2782948 (223G>A, Arg5Gln), rs11119014 (379A>G, Gln57Arg), and rs3748735 (1008G>A, Ala267Thr). The numbers of base and amino acid positions were according to NM_025179 and NP_079455, respectively.

2.3. Genotyping

Venous blood was drawn from the subjects and genomic DNA was extracted from whole blood according to the standard procedures. The SNPs were genotyped using the TaqMan 5′-exonuclease allelic discrimination assay; the assay ID (Applied Biosystems, Foster City, CA) of each SNP was C_26000091_10 for rs2782948, C_3245269_10 for rs11119014, C_3245268_20 for rs3748735, C_11673239_10 for rs2498028, rs1327175-CG for rs1327175, C_1489466_10 for rs752016, and C_8886901_1 for rs841865. Thermal cycling conditions for polymerase chain reaction (PCR) were 1 cycle at 95 °C for 10 min followed by 50 cycles of 92 °C for 15 s and 60 °C for 1 min. Genotype data were read blind to the case-control status. Ambiguous genotype data were not included in the analysis.

2.4. Statistical analysis

Deviations of genotype distributions from the Hardy–Weinberg equilibrium (HWE) were assessed with the χ^2 test for goodness of fit. Genotype and allele distributions were compared between patients and controls by using the χ^2 test for independence. These tests were performed with the SPSS software ver 11 (SPSS Japan, Tokyo, Japan). Haplotype-based association analyses were examined with the COCAPHASE software ver 2.4 (http://www.mrc-bsu.cam.ac.uk/personal/frank/software/unphased/) (Dudbridge et al., 2000). The expectation-maximization (EM) and "droprare" options were used. Haplotypes with frequencies less than 5% were considered to be rare. All P-values reported are two-tailed.

3. Results

Genotype and allele distributions of the examined SNPs of *PLXNA2* in patients and controls are shown in Table 1. The genotype distributions did not significantly deviate from the HWE in patients and controls for all SNPs except for rs3748735 (Ala267Thr) in controls. When the P-value (0.0072) of the HWE for rs3748735 in controls was corrected by Yates and Bonferroni corrections due to the very small cell counts of TT genotype and multiple testing (*i.e.*, 7 SNPs examined), respectively, the corrected P-value (0.091) was not significant anymore.

With respect to the 4 intronic SNPs (rs752016, rs841865, rs1327175, and rs2498028), there was no significant difference in genotype or allele distributions between patients and controls. Both genotype and allele distributions were approximately the same in the two groups; therefore, we failed to replicate the finding of Mah et al. (2006). For the non-synonymous polymorphisms of the *PLXNA2* gene (rs2782948, rs11119014, and rs3748735), there was again no significant difference in genotype or allele distributions between patients and controls (Table 1). We further performed stratified analysis of the data by sex. However no significant associations were observed (data not shown) for any SNP.

Results of haplotype-based analyses and Pair-wise linkage disequilibrium between neighboring SNPs are shown in Table 2. We obtained no significant difference in haplotype frequencies for any of two-, three-, four-, five-, six-, or seven-marker analyses between patients and controls.

4. Discussion

In the present study, we tried to replicate the finding of Mah et al. (2006) who reported a significant association between

PLXNA2 and schizophrenia. Furthermore, we examined 3 additional non-synonymous polymorphisms of *PLXNA2*. However, we failed to obtain any evidence for association between variants of *PLXNA2* and schizophrenia in our Japanese sample. Our sample size had a power of 90% with a 5% significance level to detect an odds ratio of 1.45 for allelic association with the SNP rs752016. There is only a small chance that a clinically meaningful difference would have been missed with the data.

Our failure to replicate the finding of Mah et al. (2006) may be attributable to ethnic differences between European Americans and Japanese. Indeed, Mah et al. (2006) obtained no evidence for significant association in their Asian American subjects with respect to the 4 intronic SNPs (rs752016, rs841865, rs1327175, and rs2498028), although this may have been attributable in part to the small number of their Asian subjects (57 patients and 71 controls). This ethnic difference might have resulted from differential linkage disequilibrium between the 4 intronic markers and functional variants responsible for giving susceptibility to schizophrenia. Indeed, based on the HapMap database (http://www.hapmap.org/) around the *PLXNA2* gene, linkage disequilibrium data between SNPs are somewhat different between Europeans and Japanese (data not shown). It is also possible that there may be differential effect of *PLXNA2* on the development of schizophrenia between the ethnic groups. Alternatively, the significant association between *PLXNA2* and schizophrenia in European Americans reported by Mah et al. (2006) may have arisen by chance. To draw any conclusion, further studies in other samples are required.

Since polymorphisms that result in an amino acid change are most likely to alter functions, we searched for non-synonymous SNPs in *PLXNA2* and examined 3 additional SNPs for association with schizophrenia. We again obtained no evidence of association for any SNP, which consolidated our failure to find evidence for association. A limitation might be the possible departure from the HWE in the control subjects for rs3748735 (uncorrected $P=0.0072$). However, the corrected P-value (0.091) was not significant any more. Furthermore, since we excluded ambiguous genotype data from the analysis, genotyping errors were unlikely. Another limitation is the differential sex distribution between the patients and controls in our sample. To resolve this issue, we compared the genotype and allelic distribution for each sex separately; however, we obtained no evidence for association with respect to any SNP in men and women. Furthermore, there were no significant difference in genotype or allele distribution for any SNP between males and females within both patients and control groups (data not shown). These ensure that the possible effect of the differential sex distribution were, if any, minimal.

In the present study, we found no evidence for association between the seven SNPs of *PLXNA2* and schizophrenia, suggesting that *PLXNA2* may not play a major role in our Japanese sample. However, we did not examine the entire region of *PLXNA2*; thus the possibility remains that other unknown SNPs in the gene may be associated in Asian schizophrenia. Furthermore, given the neurodevelopmental hypothesis of schizophrenia and the neurodevelopmental function of plexins, genes encoding plexins other than *PLXNA2* could also be candidate genes for schizophrenia.

5. Conclusion

The present study failed to confirm the findings of Mah et al. (2006), who reported a significant association between *PLXNA2* and schizophrenia. Furthermore, analysis of additional 3 non-synonymous SNPs of the gene also failed to find such an association. These results suggest that *PLXNA2* may not play a major role in the development of schizophrenia at least in the Japanese population.

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An association study of tachykinin receptor 3 gene with schizophrenia in the Japanese population

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The tachykinin receptor 3 (*TACR3*) gene encodes the neurokinin3 (NK3) receptor. Animal studies showed that agonist-induced stimulation of the NK3 receptor leads to the excessive release of dopamine in the ventral and dorsal striatal and prefrontal cortical regions. Data from clinical trials of selective NK3 receptor antagonists in schizophrenia have shown significant improvement in positive symptoms. We performed an association study of the *TACR3* gene in the Japanese population of 384 schizophrenic patients and 384 controls. Nine single nucleotide polymorphisms were genotyped using TaqMan assays and polymerase chain reaction-restriction fragment length polymorphism method. No significant association between schizophrenia and these single nucleotide polymorphisms was observed in single-marker and haplotype analyses. Our results suggest that *TACR3* is unlikely to be related to the development of schizophrenia in the Japanese population. *NeuroReport* 19:471–473 © 2008 Wolters Kluwer Health | Lippincott Williams & Wilkins.

Keywords: association study, dopamine, neurokinin3 receptor, schizophrenia, tachykinin receptor 3

Introduction

Schizophrenia is a severe, disabling and lifelong mental disorder with a global prevalence of 1%. Although it is generally accepted that genetic factors contribute to the development of this disease, its etiology has not yet been clarified.

The tachykinin receptor 3 (*TACR3*) gene located on chromosome 4q25 encodes the neurokinin 3 receptor (NK3). Its endogenous ligand is neurokinin B, a member of the tachykinin peptide family. It is expressed in the central nervous system and spinal cord [1]. Several animal studies have been performed investigating the interaction between NK3 receptors and dopamine (DA) and serotonin (5-HT) pathways. First, stimulation of NK3 receptors in the ventral mesencephalon increases DA release in the ventral and dorsal striatum and prefrontal cortex [2,3]. Second, infusion of NK3 receptor agonists into the ventral tegmental area evokes DA-mediated behaviors, such as yawning and chewing, which are potently inhibited by DA2 receptor antagonists such as haloperidol [4,5]. Third, injection of NK3 receptor agonists into the raphe area elicits 5-HT-mediated behaviors, such as head twitches, which are inhibited by 5-HT2A/5-HT2C receptor antagonists [6,7]. Additionally, a recent clinical study showed NK3 receptor antagonists improved the positive symptoms in schizophrenia [8]. These results suggest that altered form and function of the NK3 receptor might be related to the abnormalities of DA and 5-HT signaling, one of the major hypotheses explaining the pathophysiology of schizophrenia.

For all of these reasons, *TACR3* was hypothesized to be involved in the pathogenesis of schizophrenia. In this study, we performed linkage disequilibrium (LD) analysis of the *TACR3* gene and carried out case–control association studies between TACR3 polymorphisms and schizophrenia using single-marker association analysis and haplotype analysis in the Japanese population.

Methods

Participants

A total of 384 patients with schizophrenia [231 men, mean age±standard deviation (SD) 48.8±14.4; 153 women, 53.0±15.9] and 384 controls (161 men, 40.8±15.6; 223 women, 39.5±13.8) were genotyped. All participants were ethnically Japanese and unrelated to each other. The schizophrenic patients, who were diagnosed according to the DSM-IV criteria with the consensus of at least two experienced psychiatrists on the basis of empirical diagnostic interviews and review of medical records, were recruited from several psychiatric hospitals around the Tokyo and Nagoya areas (within a 350 km radius). All healthy control participants with no current or past contact with psychiatric services were also screened on the basis of brief diagnostic interviews. This study was approved by the Ethics Committee of the Nagoya University Graduate School of Medicine, and written informed consent was obtained from each participant.

Haplotype tag single nucleotide polymorphism selection
We first consulted the HapMap database (release #16c.1, *www.hapmap.org*) and determined the LD block with the criteria D' >0.8 using HAPLOVIEW ver. 3.2 software [9]. All single nucleotide polymorphisms (SNPs) listed in the entire coding region as well as the 500 bp upstream 5'-flanking region and 500 bp downstream 3'-UTR region (minor allele frequency >0.05) were included in the LD analysis. Haplotype tag SNPs (htSNPs) were defined as those capturing 90% of the haplotype diversity within each LD block using the same program. The Japanese portion of the HapMap data was used for this procedure (Fig. 1).

Single nucleotide polymorphism genotyping
Genotyping of the htSNPs was carried out using TaqMan assays (Applied Biosystems, Foster City, California, USA) and the PCR-restriction fragment length polymorphism (RFLP) method (Table 1). TaqMan probes and Universal PCR Master Mix were obtained from Applied Biosystems. A 5-μl total reaction volume was used, and allelic-specific fluorescence was measured using the ABI PRISM 7900 Sequence Detector System (Applied Biosystems). Detailed information on the PCR method is available upon request.

Statistical analysis
Genotype deviation from the Hardy–Weinberg equilibrium (HWE) was evaluated by the χ^2 test. Single marker association and haplotype analyses were performed with SPSS version 11.0J (Tokyo, Japan) and COCAPHASE version 2.403 (*http://portal.litbio.org/Registered/Option/unphased*; Dudbridge, 2003), respectively. The significance level for all statistical tests was 0.05. Power calculations were performed using the genetic statistical package Genetic Power Calculator (*http://pngu.mgh.harvard.edu/~purcell/gpc/*; Purcell 2001–2005).

Results
The *TACR3* gene was composed of six LD blocks. One nonblock SNP and eight htSNPs were finally selected according to the criteria (Fig. 1). The genotype and allele frequency of each htSNP in schizophrenic patients and controls are summarized in Table 1. The observed genotype frequencies of all SNPs were within the distribution expected according to HWE. Neither the genotype nor allele frequency of any SNP differed significantly between the schizophrenia group and the control group (Table 1). The distribution of haplotype frequencies did not differ significantly between the schizophrenic patients and controls (Table 2). More than 80% power in detecting any association with schizophrenia was obtained when the genotype relative risk was set at 1.33–1.66 under a multiplicative model of inheritance.

Discussion
Our study indicates that the *TACR3* gene does not play a major role in the development of schizophrenia in the Japanese population, as no significant differences in allele, genotype, or haplotype frequencies of the selected SNPs were found between schizophrenic patients and controls. As it is, however, suspected that genetic risk factors for schizophrenia may differ between races or ethnicities, a replication study including different ethnic populations is needed to validate these results.

As mentioned in the Introduction, the NK3 receptor was reported to regulate the DA and 5-HT release or concentration at the synapse. It would therefore be valuable to investigate the gene–gene interactions between *TACR3* and other DA or 5-HT signaling related genes [10]. Furthermore, the 5-HT1A receptor partial agonist tandospirone is reported to be effective as an adjunctive treatment to improve cognition in patients with schizophrenia [11]. As the NK3 receptor is thought to have the potential for indirect influence on the 5-HT1A receptor through 5-HT release, association analysis using samples with data on the cognitive function might help elucidate the pathogenesis of schizophrenia.

A couple of limitations in this study should be considered. First, the male/female ratios and average ages did not match between schizophrenic patients and controls. When we performed a multiple regression analysis, there were no effects of age or sex on the disease status (data not shown). Additionally, these effects might be small because not likely

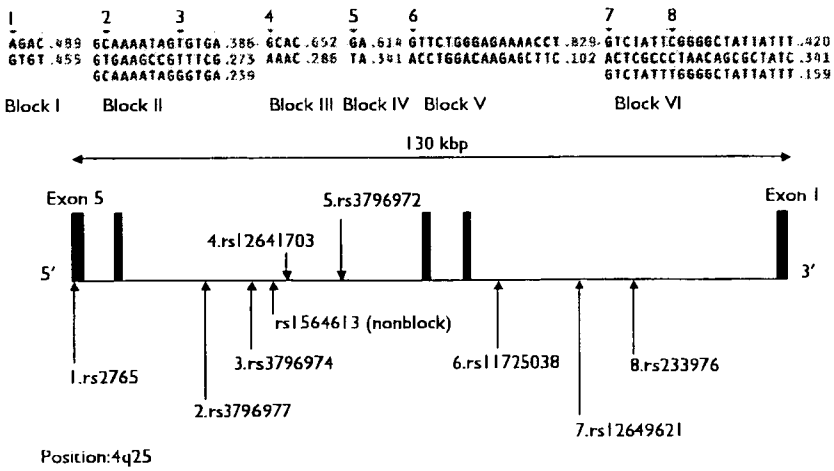

Fig. 1 Genomic structure of *TACR3* with haplotype tag single nucleotide polymorphisms (SNPs) and haplotype frequencies in each linkage disequilibrium block provided by HapMap database V.16. Numbers under or above the arrows represent the SNPs we selected in this study.

Table 1 Association analyses of haplotype tag SNPs

SNP	Block	Method of genotyping		GRR	Allelic distribution[a]			Genotypic distribution[a]			
					M	m	P value	M/M	M/m	m/m	P value
rs2765	I	TaqMan	SCZ	1.33	411	345	0.685	114	183	81	0.861
			CONT		401	351		112	178	87	
rs3796977	II	TaqMan	SCZ	1.41	611	153	0.471	249	113	20	0.79
			CONT		598	164		240	120	22	
rs3796974	II	TaqMan	SCZ	1.34	502	264	0.992	165	172	46	0.563
			CONT		493	259		169	156	52	
rs12641703	III	TaqMan	SCZ	1.35	506	260	0.669	167	172	44	0.536
			CONT		485	261		164	157	52	
rs3796972	IV	PCR-RFLP	SCZ	1.33	406	350	0.45	106	195	78	0.397
			CONT		414	330		121	177	77	
rs11725038	V	TaqMan	SCZ	1.37	556	210	0.899	204	149	31	0.954
			CONT		548	210		201	146	33	
rs12649621	VI	TaqMan	SCZ	1.33	430	336	0.284	127	178	79	0.53
			CONT		407	355		113	183	87	
rs233976	VI	TaqMan	SCZ	1.44	639	123	0.833	270	100	12	0.614
			CONT		642	120		269	106	8	
rs1564613	Non-block	TaqMan	SCZ	1.66	710	52	0.799	331	48	2	0.793
			CONT		705	49		332	43	3	

CONT, control; GRR, genotype relative risk; M, major allele; m, minor allele; SCZ, schizophrenia; SNP, single nucleotide polymorphism.
[a]In absolute numbers.

Table 2 Haplotype analyses

Block	SNP	Haplo-type	SCZ[a]	CON[a]	P value[b]	Global P value[b]
		AT	0.455	0.44	0.558	
2	rs3796977-rs3796974	AC	0.345	0.344	0.993	0.734
		CT	0.2	0.215	0.467	
		GG	0.439	0.466	0.284	
6	rs12649621-rs233976	AG	0.4	0.377	0.348	0.531
		AA	0.161	0.157	0.837	

CONT, control; SCZ, schizophrenia; SNP, single nucleotide polymorphism.
[a]Estimated frequencies.
[b]P values were calculated by log-likelihood ratio test.

more than four participants given a lifetime morbidity risk of 1% will eventually develop schizophrenia. Second, we selected htSNPs so as to cover 90% of the haplotypes within each LD block. It is, however, possible that the htSNPs used in this study did not capture all haplotypes in the gene, as the LD block structure of *TACR3* was not tight. In other words, there may be SNPs not found in the LD, for which we did not investigate the possible association with schizophrenia. Thus, further analysis based on more comprehensive and detailed SNP coverage of *TACR3* is required to make conclusive results.

Conclusion

The present results suggest that *TACR3* itself is unlikely to be related to the development of schizophrenia in the Japanese population. Further studies including pharmacogenetic investigations are required, however, for conclusive results on the exact roles of *TACR3* in the pathophysiology of schizophrenia.

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Association Study between *Apolipoprotein L* and Schizophrenia by Exhaustive and Rule-Based Combination Analysis for Identification of Multilocus Interactions

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Several single marker association and haplotypic analyses have been performed to identify susceptible genes for various common diseases, but these approaches using candidate genes did not provide accurate and consistent evidence in each analysis. This inconsistency is partly due to the fact that the common diseases are caused by complex interactions among various genetic factors. Therefore, in this study, to evaluate exhaustive genotype or allele combinations, we applied the binomial and random permutation test (BRP) proposed by Tomita *et al.* [IPSJ Digital Courier, 2, 691–709 (2006)] for the association analysis between an *Apolipoprotein L* gene cluster and schizophrenia. Using the seven selected representative single nucleotide polymorphisms (SNPs) based on the results of linkage disequilibrium evaluation, we analyzed 845 schizophrenic patients and 707 healthy controls, and investigated the validation of risk and protective factors with two randomly divided data sets. A comparative study of a method for analyzing the interactions was performed by conventional methods. Even if all the tested methods were used for analysis, the risk factor with a high significance was not commonly selected from both independent data sets. However, the significant interactions for the protective factor against disease development were commonly obtained from both data sets by BRP analysis. In conclusion, although it is considered that the causality of schizophrenia is too complex to identify a susceptible interaction using a small sample size, it was suggested that the healthy controls tend to have the same combination of certain alleles or genotypes for protection from disease development when BRP as a new exhaustive combination analytical method was used.

[**Key words:** complex genetic diseases, gene–gene interaction, single nucleotide polymorphism, binomial and random permutation test]

The HapMap project has provided valuable information on linkage disequilibrium (LD) in a particular population for elucidating genetic risk factors for common disorders (1). To date, the identification of genetic risk factors for common disorders has been successful only to a certain extent; for example, *APOE* for Alzheimer's disease (2), *NOD2* and 5q31 for inflammatory bowel disease (3), and *NRG1* for schizophrenia (4). However, these candidate genes do not provide accurate and consistent evidence in each case (*i.e.*, for review of *NRG1* [5]). Since these disorders are considered to be complex and caused by complex interactions between various genetic factors, the single marker association and haplotypic analyses cannot clarify the possibility of gene–gene interactions. Therefore, we should consider gene–gene interactions. Such interactions alter or increase the risk of complex genetic diseases in addition to the independent effects of the genes involved in such diseases, because such interactions can modify transcription or translation levels either directly or protein products indirectly (6).

Although a multidimensional approach requires the development of statistical methods that would enable us to handle multiple variable loci in different combinations, it is difficult to detect the interactions of candidate genes by traditional parametric statistical methods and case-control studies. This matter arises because it is difficult to predict complex relationships in analytical space with very few or no data points and to establish a highly dimensional prediction model from a small sample size.

To date, several analytical approaches have been proposed for gene–gene interactions (6), including logistic re-

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gression (LR) (7–9), multifactor dimensionality reduction (MDR) (10–12), artificial neural network (ANN) (13–16), S-sum statistic (17–19), and classification and regression tree (CART) (20–22). The methods that enable the statistical evaluation of one rule comprising a combination of certain alleles and genotypes with respect to both risk and protective factors (23) have been scarcely proposed to detect an interaction between genes and predict the development of a complex disease. The most important cause and effect relationship among the combinations seems to be considered as the marked rule in which the existing ratio between a case and a control is mostly biased among all rules.

In this study, we introduced a new alternative approach based on one rule and an exhaustive combination analysis to extract causal interactions with minimum errors; the approach, which is the binomial and random permutation test (BRP) (24) method, enables the automatic estimation of dominant or recessive models with respect to any exhaustive combinations and the selection of any risk and protective factor candidates composed of genotype or allele combinations. Model estimation was performed using a binomial test (25). In our method, the random permutation test (26–28) was additionally included to adjust multiple testing problems. Using the proposed method, we considered that the gene–gene interactions in the *Apolipoprotein L* (*APOL*) gene cluster are genetic susceptibility factors for schizophrenia in a Japanese population.

MATERIALS AND METHODS

Subjects, psychiatric assessment, and SNP data In this study, 96 healthy controls were recruited for the evaluation of linkage disequilibrium (LD). All subjects were unrelated to each other and were ethnically Japanese. In this study, the subjects with schizophrenia have been referred to as case subjects and the healthy controls were referred to as control subjects.

The psychiatric assessment of each subject was performed, as described in our previous paper (29). After describing the study, a written informed consent was obtained from each subject. This study was approved by the Ethnics Committee at Nagoya University and Fujita Health University. Each SNP was detected using the established method based on PCR-RFLP and TaqMan assay. None of the subjects had any missing SNP data.

The SNPs used in the evaluation of LD are listed in Table 1. LD refers to the fact that particular alleles at nearby sites can co-occur on the same haplotype more often than is expected by chance. The entire chromosome can be partitioned into high-LD regions interspersed by low-LD regions. The high-LD regions are usually called haplotype blocks and the low-LD regions are referred to as recom-

TABLE 1. Single nucleotide polymorphisms (SNPs) in linkage disequilibrium (LD) mapping and association analysis of all data and first- and second-set samples

All data SNP[a]	Genotypic association[c]										Allelic association[c]		
	MAF[b]	N		M/M		M/m		m/m		*P*-value[d]	m		*P*-value[d]
	CON	SCZ	CON	SCZ	CON	SCZ	CON	SCZ	CON		SCZ	CON	
*SNPVI-1	0.26	845	707	499	448	302	226	44	33	0.222	0.23	0.21	0.104
SNPVI-2	0.17												
SNPV-I	0.29												
*SNPV-2	0.46	845	707	271	249	400	335	174	123	0.203	0.44	0.41	0.0754
SNPIII-1	0.3												
*SNPIII-2	0.32	845	707	346	314	398	300	101	93	0.183	0.36	0.34	0.510
*SNPIV-1	0.13	845	707	619	519	210	169	16	19	0.544	0.14	0.15	0.801
*SNPIV-2	0.14	845	707	614	525	214	163	17	19	0.426	0.15	0.14	0.717
SNPIV-3	0.076												
SNPII-1	0.087												
*SNPI-1	0.13	845	707	585	484	236	207	24	16	0.678	0.17	0.17	0.942
*SNPI-2	0.42	845	707	307	260	406	330	132	117	0.827	0.40	0.40	0.891
First-set samples													
SNPVI-1		375	352	216	213	136	119	23	20	0.727	0.24	0.22	0.450
SNPV-2		375	352	109	118	182	166	84	68	0.359	0.47	0.43	0.149
SNPIII-2		375	352	150	163	187	150	38	39	0.143	0.35	0.32	0.280
SNPIV-1		375	352	289	260	80	81	6	11	0.319	0.12	0.15	0.186
SNPIV-2		375	352	285	258	84	85	6	9	0.543	0.13	0.15	0.310
SNPI-1		375	352	250	236	113	109	12	7	0.587	0.18	0.17	0.693
SNPI-2		375	352	126	132	195	159	54	61	0.174	0.40	0.40	0.850
Second-set samples													
SNPVI-1		470	355	283	235	166	107	21	13	0.211	0.22	0.19	0.0917
SNPV-2		470	355	162	131	218	169	90	55	0.379	0.42	0.39	0.213
SNPIII-2		470	355	196	151	211	150	63	54	0.663	0.36	0.36	0.838
SNPIV-1		470	355	330	259	130	88	10	8	0.651	0.16	0.15	0.466
SNPIV-2		470	355	329	267	130	78	11	10	0.171	0.16	0.14	0.184
SNPI-1		470	355	335	248	123	98	12	9	0.899	0.16	0.16	0.701
SNPI-2		470	355	181	128	211	171	78	56	0.645	0.39	0.40	0.737

[a] SNPs denoted by an asterisk in all data indicate representative SNPs.
[b] MAF = minor allele frequency of 96 samples. MAF is the frequency of minor allele m, which is determined by counting two alternative alleles within a sample.
[c] N, Number of subjects; M, major allele; m, minor allele; SCZ, schizophrenics; and CON, controls.
[d] *P* value was calculated using the χ^2 test.

TABLE 2. Two-locus haplotype frequencies for the evaluation of linkage disequilibrium (LD)

		Locus 2	
		Allele M	Allele m
Locus 1	Allele M	a	b
	Allele m	c	d

bination hotspots. Within a haplotype block, there is little or no recombination that occurs and the SNPs are highly correlated. Consequently, a small subset of SNPs (called representative SNPs or tag SNPs) is sufficient to capture the haplotype pattern of the block. Thus, in order to obtain SNPs to provide the enough information required for combination analysis, we first performed one representative SNP selection in one LD block. The D′ value is one of the measures for assessing the strength of LD ranging from 0 (no disequilibrium) to 1 (complete disequilibrium). The criterion of an LD block is to be a region in which all pairwise D′ values are not lower than 0.8, using the Genotype2LDblock v0.2 software (30). The estimation of relative two-locus haplotype frequencies is performed using Table 2. The frequencies of haplotypes MM, Mm, mM and mm in loci 1 and 2 are defined as the values a, b, c, and d, respectively. D is defined as (ad)−(bc) ranging from $D_{max} = \min\{(a+b)(b+d), (c+d)(a+c)\}$ to $D_{min} = \max\{-(a+b)(a+c), -(c+d)(b+d)\}$. D′ is defined as D/D_{max} for $D>0$ and as D/D_{min} for $D<0$ to change D to D′ ranging from 0 to 1. From each LD block, we selected representative SNPs (denoted by an asterisk in Table 1) with the highest minor allele frequencies (MAFs). MAF is (c+d) and (b+d) in loci 1 and 2, respectively. The higher the MAF is, the more information the SNP has, compared with the other SNPs in the same LD block for identifying factors susceptible to diseases because of subject variation.

SNP association analysis To assess the association of an SNP with a disease, the χ^2 test based on genotypic and allelic association analyses was performed in all data and in the first- and second-set samples divided randomly, as shown in Table 1. The number of subjects (N) belonging to each category composed of phenotypes (schizophrenia or control) and genotypes or alleles, and the P value calculated using the χ^2 test are shown in Table 1. We employed this validation test using two independent data sets (referred to as the first- and second-set samples), because commonly selected significant factors might have the potential as factors susceptible to schizophrenia. Therefore, these two data sets indicate a relationship between modeling and test data sets, which are generally used for validation analysis. The extents of genotypic and allelic associations were measured from the P value calculated using the χ^2 test in the 2 (case or control)×3 (genotype; M/M, M/m and m/m) and 2 (case or control)×2 (allelic association, M or m) tables, respectively.

BRP analysis The concept of the BRP test (24) is based on the evaluation of the interactions between several factors by statistically assessing the extent of bias in the number of case or control subjects belonging to one rule comprising a combination of certain alleles or genotypes. A rule table constructed to analyze two SNPs (SNPs A and B) with a dominant model and a recessive model, respectively is shown in Fig. 1. One cell in Fig. 1 corresponds to one rule; thus, there are four and eight rules in the cases of two and three SNP combinations, respectively. For example, in rule no. 1 in Fig. 1, one of the rules in using the two-SNP combination, subjects with the genotype AA of SNP A and the B allele of SNP B belong to the rule. In each rule, the extent of bias in the number of case or control subjects (the ratio between them) was assessed using the BRP test mentioned below. We focused the rule with statistically biased ratios between the case and control subjects in either the first- or second-set samples. Thus, the rules consisting of more

		SNP A	
		AA	Aa + aa
SNP B	BB + Bb	$N_{case,1}/N_{control,1}$	$N_{case,2}/N_{control,2}$
	bb	$N_{case,3}/N_{control,3}$	$N_{case,4}/N_{control,4}$

FIG. 1. Rule table using combination of two single nucleotide polymorphisms (SNPs). $N_{case,i}$ and $N_{control,i}$ represent the numbers of case and control subjects, respectively, belonging to rule no. i. The rule circled by a dotted line denotes subjects with the genotype AA of SNP A and the B allele of SNP B. This rule is regarded as rule no. 1.

case subjects are regarded as risk factor candidates (RFCs) and the rules with control subjects are regarded as protective factor candidates (PFCs). In addition, if the rules have statistically biased ratios between case and control subjects in both data sets, they are defined as risk or protective factors.

We applied the binomial test (25) to the combination analysis and selected RFCs or PFCs. The binominal test is used for the evaluation of the potentiality of a risk or protective factor. The P value of the binomial test for evaluating the existing ratio between the case and control subjects is calculated using the binomial distribution as

$$f(N_{case,i}) = \frac{n!}{N_{case,i}!\,(n-N_{case,i})!}\, p^{N_{case,i}}(1-p)^{n-N_{case,i}} \quad (1)$$

where n is the sum of the observed $N_{case,i}$ and $N_{control,i}$ existing in rule i. The probability p represents $N_{case}/(N_{case}+N_{control})$, where N_{case} and $N_{control}$ represent the total numbers of cases and controls analyzed in the combination. The null hypothesis ($N_{case,i}/N_{case} \le N_{control,i}/N_{control}$) in the case of selecting RFCs is tested by computing the sum (P value) of all $f(N_{case,i})$ that are equal to or lesser than that for the observed $N_{case,i}$ (one-tailed test) (24, 25).

In addition, to adjust for the multiple testing problems caused by a simultaneous significance test, a random permutation test (26–28) was performed in this method. The procedure of the BRP analysis is outlined in Fig. 2 and has two steps. This procedure with two steps was repeated twice in the selection of RFCs and PFCs. The exhaustive combinations of loci were analyzed in this method; therefore, when using g SNPs, the number of combinations N_{comb} is given as

$$N_{comb} = \sum_{i=1}^{g} {}_gC_i \quad (2)$$

In step 1, the most efficacious genotype combination in each SNP combination is determined as follows. In the case of the two-SNP combination, there are 16 P values calculated using the binomial test in four genotype combinations: dominant-dominant, dominant-recessive, recessive-dominant and recessive-recessive (each combination has four rules). By comparing these P values under the condition $N_{case,i}/N_{case} > N_{control,i}/N_{control}$, the combination of dominant and recessive is determined when the lowest P value is obtained in the exhaustive genotype combinations. (In the case of selecting PFCs, the condition used is $N_{case,i}/N_{case} < N_{control,i}/N_{control}$.) N_{case} and $N_{control}$ represent the numbers of case and control subjects analyzed in the combination, respectively. Next, the P values in the acquired genotype combination are used in the selection of RFCs or PFCs (step 2). In this study, since the method of SNP analysis using dominant

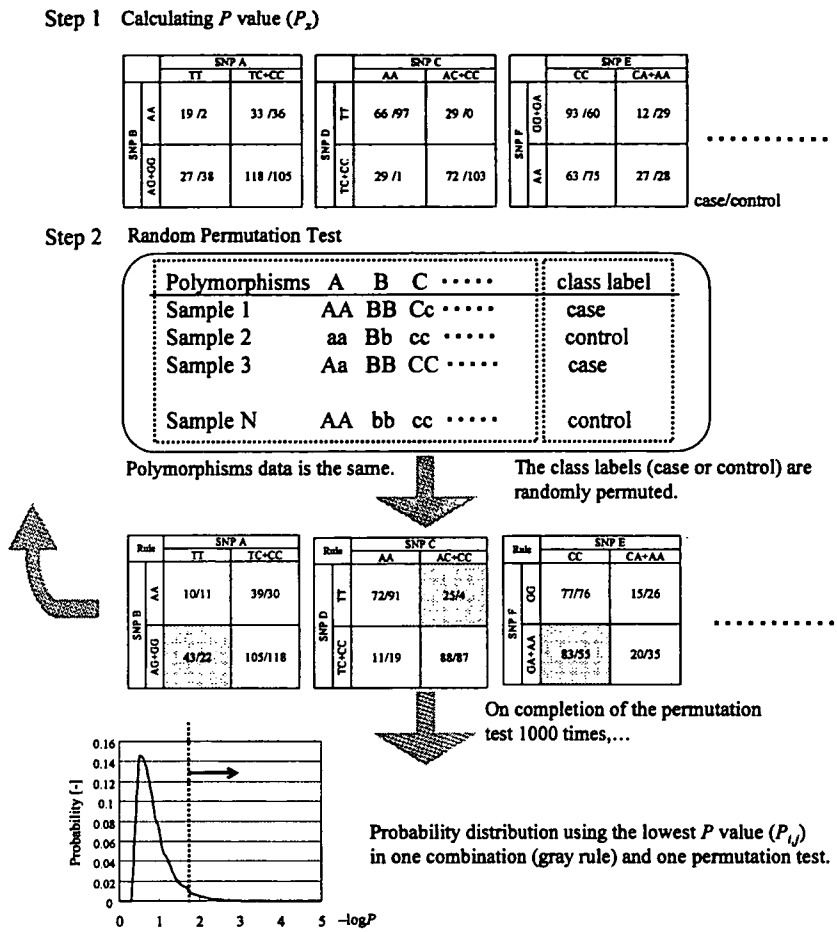

FIG. 2. Selection procedure for risk factor candidates (RFCs) using binomial and random permutation test (BRP) in combination of two polymorphisms. The analysis procedure has been divided into two steps. In step 1, the P values in all rules (under the condition $N_{case,i}/N_{case} > N_{control,i}/N_{control}$) are calculated for the genotype combinations of the dominant and recessive defined by the following condition. The combination of dominant and recessive is determined when the P value in one of the rules under the condition $N_{case,i}/N_{case} > N_{control,i}/N_{control}$ is the lowest among the P values in exhaustive genotype combinations. In step 2, to select the RFCs, the statistical significance of the rule in each combination is assigned to the P value ($P^{ran}(P_x)$) that is calculated using the P value (P_x) derived from step 1 by modeling the null distribution; the P value comprising the null distribution is the lowest ($P_{i,j}$ in Eq. 3) under the condition $N_{case,i}/N_{case} > N_{control,i}/N_{control}$ in each combination (gray rule) by the random permutation test. In the random permutation test, the signal of each subject (case or control) is randomized to avoid a change in the number of subjects contained in the rule; in the present study, the number of permutations was 1000 times. RFCs were inferred at the $P^{ran}(P_x)$ obtained using the distribution and was calculated to be smaller than 0.05 ($P^{ran}(P_x)<0.05$).

and recessive concepts appears practical for the application of various phenotypes (such as diseases), the heterozygote is combined with either of the homozygotes as described above. The dominant model is determined by comparing the Aa plus aa genotypes with the AA genotype, and the recessive model is determined by comparing the aa genotype with the AA plus Aa genotypes.

In step 2, to select RFCs (or PFCs), the statistical significance of the rule in each combination is denoted by the P value ($P^{ran}(P_x)$) calculated using the P value derived from step 1 by modeling the null distribution; the P value comprising the null distribution is the lowest under the condition $N_{case,i}/N_{case} > N_{control,i}/N_{control}$ in each combination using the random permutation test (26–28). (In the case of selecting PFCs, the condition used is $N_{case,i}/N_{case} < N_{control,i}/N_{control}$.) This leads to the development of a procedure for determining the ratio between the case and control subjects, which is statistically significant when compared with the null hypothesis of the ratio in randomly labeled data. The null hypothesis indicates that given a particular rule (r), the conditional probability of a label (y) being case (+1) and that of a label (y) being control (−1) are equal as

$$H_0: p(y=+1|r) = p(y=-1|r)$$

In the random permutation test, the label of each subject (case or control) is randomized to inhibit a change in the number of subjects contained in the rule. In essence, we can examine how well the rule of correctly labeled data in each combination explains the extent of risk (or protection) in comparison with the rule of randomly labeled data. The significance of the rule is $P^{ran}(P_x)$ (Eq. 3), which is a percentage of random rules (27).

$$P^{ran}(P_x) = \frac{1}{T_1 \times T_2} \sum_{i=1}^{T_1} \sum_{j=1}^{T_2} \theta(P_x - P_{i,j}) \quad (3)$$

Here, $\theta(z)=1$ if $z \geq 0$ and 0 otherwise. $P_{i,j}$ is the lowest P value of the rule obtained using randomly labeled data calculated using the binomial test in one combination and one permutation test. P_x is the P value of the rule obtained using correctly labeled data calculated using the binomial test in step 1. In other words, $P^{ran}(P_x)$ is

the P value of P_x in the null distribution. T_1 and T_2 are the numbers of permutations and combinations, respectively. T_1 is 1000 times in this study. T_2, for example, in two-SNP combinations using seven SNPs is ${}_7C_2=21$, because in the random permutation test, the combination of dominant and recessive is already determined using correctly labeled data as mentioned above. In this study, RFCs and PFCs are inferred at the $P^{ran}(P_x)$ level using the distribution calculated using the random permutation test and are found to be less than 0.05 ($P^{ran}(P_x)<0.05$). The BRP software is available at http://www.nubio.nagoya-u.ac.jp/proc/english/indexe.htm.

In addition, our proposed BRP method was compared with MDR and S-sum statistic, because these two methods enable the evaluation of interactions between SNPs using the P value based on the results of the random permutation test. The null distribution used for calculating the P value is different among these methods. MDR (11) enables the evaluation of ratios between case and control subjects in all rules in one combination of SNPs and the calculation of the testing accuracies of exhaustive combinations (Eq. 2) in the 10-fold cross validation. MDR was also assessed from the cross-validation consistency and P value computed by comparing its (accuracy or consistency) value with the empirical distribution (random permutation test) (11). The null distribution is determined using the testing accuracy in randomly labeled data. In S-sum statistic (17), SNPs were added to the model stepwise according to their S-value ranked highest ($S=\Sigma_i(t_i\times u_i)$, where t_i is the χ^2 value that enables the evaluation of allelic association in the 2×2 table and u_i is the Hardy-Weinberg disequilibrium for association in the ith SNP), that is, their contribution to the disease risk. SNPs reducing the P value estimated using the permutation test in each sum to a minimum provides information regarding the significant SNP combination and the number of SNPs in the analyzed SNPs. The null distribution is determined using the S value in randomly labeled data (17).

RESULTS

Interaction analysis of complex genetic diseases using BRP First, the association between the isolated SNPs and schizophrenia was assessed from the P value calculated using the χ^2 test with respect to the genotypic and allelic data. As shown in Table 1, there was no association between the isolated SNPs and schizophrenia in the genotypic and allelic analyses. Therefore, we focused on the analysis of SNP combinations. To validate risk or protective factor candidates (RFCs or PFCs), the BRP analysis (24) was performed by dividing the original data to two data sets (first- and second-set samples) randomly. In Fig. 3, rules (up to a three-SNP combination) with a higher control subject rate ($N_{control,i}/(N_{case,i}+N_{control,i})$) than that of population data were plotted in the more than 0 area on the $-\log P_b$ axis, whereas those with a higher case subject rate ($N_{case,i}/(N_{case,i}+N_{control,i})$) than that of population data were plotted in the less than 0 area on the $-\log(1/P_b)$ axis. P_b was calculated with the binomial test. The black dot in Fig. 3 represents the RFC or PFC rule in which $P^{ran}(P_x)$ is smaller than 0.05 in both data sets. Although the validation of the RFC could not be found out in this sample size, several rules for the control showed the same tendency of the P value between both data sets, on the basis of the rules plotted in the more than 0 area on the $-\log P_b$ axis in both data sets. In addition, four identical PFCs (protective factors) were obtained, as shown in Fig. 3. As shown in Table 3 and Fig. 4, the protective factors were obtained by combining the GG genotypes of SNP VI-1 and SNP V-2. Because the ratio between the case and control subjects in the protective factor comprising these two genotypes patterns is statistically significant in both data sets divided randomly, the evidence indicating that the distribution of subjects who have this genotype combination in the present data might be the same as that of population data was obtained in the present sample size by assessing the ratio in one rule using BRP.

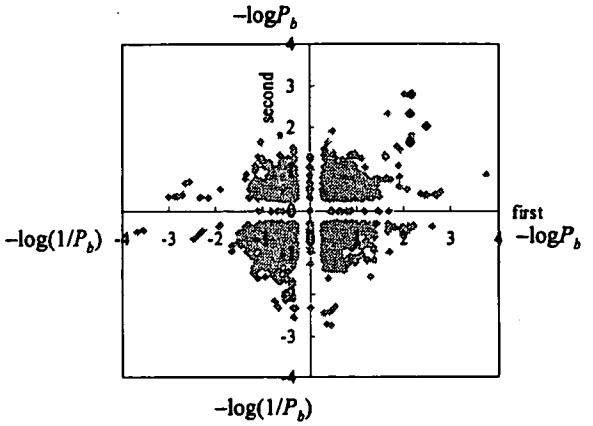

FIG. 3. Relationship of P value in same rule between first- and second-set samples in up to three single nucleotide polymorphism (SNP) combinations. Rules with a higher control subject rate than that of population data were plotted in the more than 0 area on the $-\log P_b$ axis, whereas those with a higher case subject rate than that of population data were plotted in the less than 0 area on the $-\log(1/P_b)$ axis. P_b was calculated with the binomial test. The black dot represents the rule in which the P value ($P^{ran}(P_x)$) is smaller than 0.05 in both data sets.

TABLE 3. Protective factors against disease development obtained in first- and second-set samples by BRP analysis

SNP			P^{ran} [a]	
			First set	Second set
VI-1	V-2		0.0104	0.0442
VI-1	V-2	IV-1	0.0195	0.0065
VI-1	V-2	IV-2	0.0201	0.0194
VI-1	V-2	I-1	0.0083	0.0417

[a] The P value was calculated with the binomial and random permutation test (BRP). The P values of the protective factors were smaller than 0.05 ($P^{ran}(P_x)<0.05$) in both data set samples.

Comparative study of interaction analysis with BRP and screening signal SNPs Next, in order to investigate the performance of BRP in the gene–gene interaction analysis, we compared BRP with MDR and S-sum statistic that enable the evaluation of a gene–gene interaction using the P value based on the results of the random permutation test. In Table 4, with the best models in each data set evaluated on the basis of the testing accuracy, cross-validation consistency and P value in each number of input variables, the same significant interactions were not observed by MDR. An interaction effect was not observed in the S-sum statistic analysis using the P value (0.285 and 0.792 in the first- and second-set samples, respectively).

DISCUSSION

Schizophrenia is a neurodevelopmental disorder and one

First-set samples

		SNP VI-1	
		GG	GA + AA
SNP V-2	GG	61 / 86 $P_b = 0.0072$	48 / 32
	GC + CC	155 / 127	111 / 107

case / control

Second-set samples

		SNP VI-1	
		GG	GA + AA
SNP V-2	GG	94 / 95 $P_b = 0.0223$	68 / 36
	GC + CC	189 / 140	119 / 84

case / control

FIG. 4. Protective factor (gray cell) in both first- and second-set samples. P_b was calculated with the binomial test.

of the common diseases with an estimated heritability of 80%. Chromosome 22q11–q13 (OMIM: #600850 SCZD4) is one of the most probable schizophrenia susceptibility regions because the microdeletions of the 22q11 chromosome are reported to be associated with schizophrenia (31); furthermore, the two independent meta-analyses of linkage studies reveal the suggested linkage in this region (32, 33).

The APOL proteins belong to a group of high-density lipoproteins (HDL), and all 6 *APOL* genes (*APOL1–6*) are located near each other on the 22q12 chromosome. A recent postmortem study using the candidate gene cDNA array showed the upregulations of *APOL1*, *APOL2*, and *APOL4* in the prefrontal cortex of schizophrenic patients (34).

The most recent association analysis using the DNA pooling method showed no association of this gene cluster with schizophrenia in Irish patients (35). Moreover, the single marker association analysis (*i.e.*, allelic and genotypic analyses), as well as haplotypic association analysis, could not confirm the possibility of gene–gene interactions. In the present study, we performed the BRP analysis (24) to evaluate the association of the *APOL* gene cluster with schizophrenia (interaction) in a Japanese population by focusing on the analysis of SNP combinations that should not be within the same LD block. By the combination analysis using BRP, we considered that the interactions among SNPs in the *APOL* gene cluster are genetic susceptibility factors for schizophrenia.

In this validation analysis, although the same risk factor could not be selected in the two independent data sets, a combination of the GG genotypes of SNP VI-1 and SNP V-2 was selected as a significant protective factor against disease development in both data sets (Fig. 4). This result indicates the possibility that the distribution of subjects who have this genotype combination in the present data might be the same as that of population data because of the concordance between the two independent data sets. However, this significant protective factor was not detected in the MDR and S-sum statistic analyses, because these approaches do not enable the evaluation of the ratio between the case and control subjects in one rule. The ratio in rules, except risk or protective factors, seems to indicate the same tendency as that in randomly labeled data and these rules can be ex-

TABLE 4. Results of multifactor dimensionality reduction (MDR) analysis in first- and second-set samples

Number of input variable[a]	VI-1	V-2	III-2	IV-1	IV-2	I-1	I-2	Training accuracy[b]	Testing accuracy[b]	*P* value[c]	Cross validation consistency[d]
First-set samples											
1			**[e]					0.537	0.488	0.992	5/10
2	*	*						0.558	0.502	0.950	5/10
3	*	*					*	0.586	0.479	0.998	5/10
4	*	*	*				*	0.625	0.490	0.987	8/10
5	*	*	*		*		*	0.670	0.518	0.806	8/10
6	*	*	*		*	*	*	0.715	0.491	0.983	10/10
7	*	*	*	*	*	*	*	0.749	0.506	0.921	10/10
Second-set samples											
1	*							0.570	0.560	0.889	6/10
2	*		*					0.576	0.543	1.000	2/10
3	*	*		*				0.597	0.539	1.000	6/10
4	*	*	*			*		0.628	0.505	1.000	3/10
5	*	*	*		*		*	0.664	0.510	1.000	4/10
6	*	*	*		*	*	*	0.705	0.509	1.000	8/10
7	*	*	*	*	*	*	*	0.740	0.519	1.000	10/10

[a] The model with the lowest prediction error and highest cross validation consistency was selected for each of the input variables considered.
[b] Ratio of correct classifications to total number of instances classified through 10-fold cross validation within training or testing data set.
[c] An empirical *P* value for the result was determined using 1000-fold random permutation test strategies.
[d] Frequency of times in particular cross-validated run for which given input variable combination was selected as best model.
[e] SNP was used as the input variable in the model.

cluded using BRP based on the random permutation test. Because MDR enables the simultaneous evaluation of the ratios in all rules and the testing accuracy (11), it might miss the significant rule. In S-sum statistics, the χ^2 values of allelic association and Hardy-Weinberg disequilibrium in each SNP are added simply (17); thus, the ratio obtained by combining several SNPs is not considered. It is suggested that this rule selected using BRP could be the sole protective factor against disease development with a statistical significance and is a preventable factor for schizophrenia discussed below. There are 336 subjects with this genotype corresponding to this rule in this study. These 336 subjects consist of 155 cases and 181 controls, and correspond to 25.6% of all the controls. Although the number of subjects corresponding to the rule is small, the odd ratio is significantly high (1.5).

The conventional argument is that the identification of susceptible genes leads to the discovery of new therapeutics and diagnoses. However, the occurrence of healthy individuals who can prevent disease development, despite the presence of genetic and environmental risks, increases the possibility that protective alleles or genotypes (protective factors) maintain good health (36). These protective factors probably prevent the development of disease effectively and safely. This finding seems to be the same as the following example; people who are active in immune response can avoid a viral infection, which is generally desirable. Therefore, even if subjects have the same genotype or allele combination corresponding to a risk factor, some of these subjects are considered controls because a protective factor functions. On the other hand, it is likely that there are important and inevitable protective factors; if the protective factor has been destroyed or inactivated, a disease will develop at a high probability. By using BRP that enables the evaluation of all rules exhaustively, this protective factor was selected in both independent data sets. Thus, in complex genetic diseases, such as schizophrenia, the evidence indicating that healthy control subjects tend to have the same combinations of certain alleles and genotypes was obtained using BRP. With respect to the risk factor, the causality of schizophrenia is too complex to identify a susceptible interaction using a small sample size because there might be many development patterns and differs on an individual basis.

Thus, we could not find any risk factor which can explain the biological mechanism of disease development in all patients. Owing to this result, it is likely that there might be more biological routes for disease, namely, it is considered that there might be the effects of confounding factors (such as age, sex, environmental factors and other genes) for disease development except seven SNPs analyzed in this study. In selecting risk factors, because the effects of these susceptible confounding factors might differ between independent data sets, a common risk factor might not be selected despite its significance in either data set. However, in selecting protective factors, because the effects of these factors might be small between data sets compared with the case of selecting risk factors, a protective factor might be selected using a comparatively small sample size.

For achieving a high power in selecting the protective factor, the BRP analysis has three characteristic features: (i) exhaustive combination analysis, (ii) the automatic assessment of the dominant or recessive model, and (iii) the statistical evaluation of the ratio between the case and control subjects in one rule comprising genotype or allele combinations using the binomial and random permutation tests. The first feature ensures the analysis of all possible combinations and thus helps in finding the synergistic interaction effects required for the development of a complex genetic disease. Using the second feature, the data in high dimensions obtained by combining three genotype patterns can be transformed to those in low dimensions using dominant-recessive combinations. Furthermore, from this information, important evidence on the biological aspects and the extent of risk in one rule determined from the P value calculated with BRP, which is a one-dimensional analysis (risk or non risk), might be obtained. The third feature enables us to determine how well the rule of correctly labeled data in each combination explains the extent of risk or protection compared with the rule of randomly labeled data; thus, statistically significant risk or protective factors can be obtained. Consequently, BRP can be a more effective tool than MDR and S-sum statistic because of the three features mentioned above. To clarify the pathophysiology of complex genetic diseases or the mechanism of treatment response, it is very important to identify the protective factor comprising a combination of certain alleles and genotypes, as well as the risk factor.

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The Dysbindin Gene (*DTNBP1*) Is Associated with Methamphetamine Psychosis

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Background: The dysbindin (*DTNBP1* [dystrobrevin-binding protein 1]) gene has repeatedly been shown to be associated with schizophrenia across diverse populations. One study also showed that risk haplotypes were shared with a bipolar disorder subgroup with psychotic episodes, but not with all cases. *DTNBP1* may confer susceptibility to psychotic symptoms in various psychiatric disorders besides schizophrenia.

Methods: Methamphetamine psychosis, the psychotic symptoms of which are close to those observed in schizophrenia, was investigated through a case (n = 197)–control (n = 243) association analyses of *DTNBP1*.

Results: *DTNBP1* showed significant associations with methamphetamine psychosis at polymorphisms of P1635 (rs3213207, $p = .00003$) and SNPA (rs2619538, $p = .049$) and the three-locus haplotype of P1655 (rs2619539)-P1635-SNPA (permutation $p = .0005$). The C-A-A haplotype, which was identical to the protective haplotype previously reported for schizophrenia and psychotic bipolar disorders, was a protective factor ($p = .0013$, odds ratio [OR] = .62, 95% confidence interval [CI] .51–.77) for methamphetamine psychosis. The C-G-T haplotype was a risk for methamphetamine psychosis ($p = .0012$, OR = 14.9, 95% CI 3.5–64.2).

Conclusions: Our genetic evidence suggests that *DTNBP1* is involved in psychotic liability not only for schizophrenia but also for other psychotic disorders, including substance-induced psychosis.

Key Words: Akt1, *DTNBP1*, dysbindin, methamphetamine psychosis, substance dependence

A genetic variation of the dystrobrevin-binding protein 1 (*DTNBP1*) gene has recently been shown to be associated with schizophrenia in several independent studies. Straub *et al.* (1) revealed original evidence for a positive genetic association between schizophrenia and variants in a gene on 6p22.3, dysbindin (*DTNBP1*), which is located within one of several promising loci revealed by a genomewide linkage scan. Many replication studies showed consistent findings in different populations, for example, German (2), Irish (3), Chinese (4), Swedish/German/Polish (5), UK/Irish (5), Bulgarian (6), American (7), Scottish/Chinese (8), and Japanese (9), although the significantly associated alleles and haplotypes were not always consistent among populations. Two postmortem studies also revealed that dysbindin protein or its mRNA level was reduced in the dorsolateral prefrontal cortex and in presynaptic glutamatergic terminals of the hippocampus of schizophrenia patients (10,11). These findings suggest that the dysbindin is involved in the pathogenesis of schizophrenia.

Recently, Raybould *et al.* (12) examined three loci of the *DTNBP1* gene in a large sample of patients with bipolar disorder, another endogenous psychosis, in UK Caucasians, and found that the *DTNBP1* gene was not associated with all cases of bipolar disorder but was associated with a subgroup of bipolar disorder characterized by the complication of psychotic features during episodes. The risk and protective haplotype were identical to those found in their previous schizophrenia study (13). Therefore, they speculated that the *DTNBP1* genetic variation influences susceptibility to schizophrenia and bipolar psychosis across the Kraepelinian dichotomy.

Abuse of large amounts of methamphetamine for long periods easily produces psychotic symptoms, such as delusions of reference, persecution, and poisoning, as well as auditory and visual hallucinations (14–16). Further consumption of methamphetamine may result in severe psychosis, liability to relapse with reconsumption of methamphetamine or psychological stress, and a gradually worsening prognosis. Clinical similarities between methamphetamine psychosis and schizophrenia in a cross-section of clinical features have been noted; these include auditory hallucination and delusion, the longitudinal process of progressive exacerbation with acute relapses, relatively good response to neuroleptics, and enduring vulnerability to relapse to stressors, especially in the paranoid type of schizophrenia. Indeed, methamphetamine psychosis has long been considered a pharmacologic model of schizophrenia (17,18), and shared molecular mechanisms could be involved in these psychotic disorders. Based on this rationale, it is possible that the *DTNBP1*

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gene may influence susceptibility to substance-induced psychoses in the same manner that influence susceptibility to schizophrenia and bipolar psychosis disorders. To examine this hypothesis, we investigated the association between *DTNBP1* and methamphetamine psychosis in a case–control analyses.

Methods and Materials

Subjects

The subjects consisted of 197 patients (162 male, 35 female; mean age ± SD, 38.1 ± 12.6) with methamphetamine psychosis (MAP) and 243 age-, gender-, and geographic-origin-matched healthy control subjects (193 male, 50 female; mean age ± SD, 37.2 ± 12.0) who had no individual or family history of drug dependence or major psychotic disorders such as schizophrenia and bipolar disorders. All the subjects were unrelated Japanese who were born and lived in relatively restricted areas of Japan. All patients were outpatients or inpatients in psychiatric hospitals of the Japanese Genetics Initiative for Drug Abuse (JGIDA). Consensus diagnoses of methamphetamine psychosis were made by two trained psychiatrists according to the ICD-10 criteria on the basis of unstructured interviews and medical records. All healthy control subjects were also psychiatrically screened based on unstructured interviews. The study protocol and purpose were explained to all subjects participating in the study, and written informed consent was obtained from all subjects. This study was approved by the Ethics Committee of each participating institute of JGIDA.

The patients with methamphetamine psychosis were divided into subgroups according to three clinical phenotypes that may indirectly indicate the severity of and liability to psychosis:

1. *Latency to onset of psychotic state after initial methamphetamine consumption:* Median latency was 3 years; 99 (54.4%) of patients developed psychotic symptoms within 3 years of the first methamphetamine abuse, and 83 (45.6%) patients did after 3 or more years.
2. *Duration of the psychotic state after therapy:* Methamphetamine-induced psychosis (transient type) will usually subside within 10 days to 1 month following discontinuance of consumption and beginning of pharmacologic therapy with antipsychotics such as haloperidol or risperidone. Some patients show sustained (longer than 1 month) psychotic symptoms (prolonged type), however, regardless of detoxification from methamphetamine and adequate antipsychotic therapy (16,19). In our study, 107 (56.6%) patients showed the transient type of psychosis, and 82 (43.4%) patients showed the prolonged type of psychosis.
3. *Complication of spontaneous psychosis:* Once methamphetamine psychosis has developed, some remitted patients may experience spontaneous relapse due to nonspecific stresses, such as severe fatigue or life problems, without consumption of methamphetamine. The observation period for the presence or absence of spontaneous relapse was at least 1 year and averaged 12.3 ± 11.1 years. Eighty-three patients (42.8%) experienced spontaneous relapse, and 111 (57.2%) did not.

As to multisubstance abuse status, 37.2% patients concurrently abused other illicit drugs in addition to methamphetamine. Cannabinoids were most frequently abused (34.0%), followed by LSD (14.1%), cocaine (13.1%), opioids (12%), and hypnotics (9.9%). More than 60% of patients abused only methamphetamine, but about half had a past history of organic solvent abuse in their teenage years. All clinical data were obtained from interviews with patients and their families. Urine examination was not applied.

DNA Analysis

We genotyped the three single nucleotide polymorphisms (SNPs), P1655 (rs2619539), P1635 (rs3213207), and SNPA (rs2619538) of the *DTNBP1* gene that were examined previously by O'Donovan's group and were shown to have a significant association with both schizophrenia and psychotic bipolar disorders (12,13). They showed in the schizophrenia study that these three locus haplotypes showed the most significant results among 26 significantly associated haplotypes constructed by combinations of 9 SNPs of *DTNBP*. P1655 and P1635 were two of the markers that had provided the most significant results in the study by Straub *et al.* (1), and SNPA was reported to be significantly associated with schizophrenia in a Japanese population (9).

The genomic DNA was extracted from peripheral leukocytes using the phenolchloroform method. Genotyping was performed by the polymerase chain reaction (PCR)–restriction fragment length polymorphism method. Each polymorphic site was amplified by PCR in a volume of 15 mL containing 3% dimethyl sulfoxide and .75 units of Taq DNA polymerase (Promega, Japan) using a unique primer set (P1655 [mismatch]; 5′-ATCAGGCAAAATGATGTACTGC-3′, 5′-GCCTTTTAAATAATCCTATTAGCTATGAGAGT-3′, P1635; 5′-CTTTATGCAATAAGTATTCCTG-3′, 5′-GTATACCCTGTTTTAAGCAGAC-3′, SNPA; 5′-CCTGTTTCTCAACTTAGTACAC-3′, 5′-CCTTTATCTTATTTAACTCCTG-3′). PCR reaction was performed under the following conditions: 95°C for 5 min, then 35 denaturing cycles of 30 sec each at 95°C, 1 min of annealing at the appropriate temperature, and 30 sec of extension, and final elongation at 72°C for 10 min. The PCR products were digested with the corresponding restriction enzyme for each polymorphism, *Hin*fI for P1655, *Bse*NI for P1635, and *Cai*I for SNPA, and then electrophoresed on 3.0% agarose gels and stained with ethidium bromide. All genotyping was performed in a blinded fashion, with the control and case samples mixed randomly. Part of the genotyping of P1655, P1635, and SNPA was confirmed by direct sequencing and a TaqMan SNP genotyping assay (C_16036968_10), respectively.

Statistical Analysis

Statistical analysis of association was performed using SNPAlyze software (Dynacom, Mobara City, Chiba, Japan). Deviation from Hardy–Weinberg equilibrium and the case–control study were tested using the χ^2 test. Linkage disequilibrium (LD) was tested using the χ^2 test, and D' and r^2 values were made the index in the authorization of LD. Case–control haplotype analysis was performed by the permutation method, and permutation p values were calculated based on 100,000 replications.

GenBank/EMBL Accession Numbers

Genome; NC_000006.10, NT_007592.14, MIM; 607145.

Results

The genotype distribution and allele frequencies for each polymorphism of patients with methamphetamine psychosis and control subjects are shown in Table 1. The genotype distributions of patients and control subjects did not deviate from the Hardy–Weinberg equilibrium at any of the three SNPs. We found a significant difference between patients and control subjects in the frequencies of the genotype or allele at P1635 and SNPA of

Table 1. Genotype and Allele Distribution of Three Single Nucleotide Polymorphisms of the *DTNBP1* Gene in Control Subjects and Patients with Methamphetamine (MAP) Psychosis

			Genotype				Allele		
		N	C/C	C/G	G/G	*p*	C	G	*p*
P1655	rs2619539								
Control		240	118 (49.2)	107 (44.6)	15 (6.2)		343 (71.5)	137 (28.5)	
MAP Psychosis		190	78 (41.0)	94 (49.5)	18 (9.5)	.17	250 (65.8)	130 (34.2)	.076
P1635	rs3213207		A/A	A/G	G/G		A	G	
Control		243	239 (98.4)	4 (1.6)	0 (.0)		482 (99.2)	4 (.8)	
MAP Psychosis		197	175 (88.8)	22 (11.2)	0 (.0)	.000025	372 (94.4)	22 (5.6)	.000030
SNPA	rs2619538		A/A	A/T	T/T		A	T	
Control		232	225 (97.0)	7 (3.0)	0 (.0)		457 (98.5)	7 (1.5)	
MAP Psychosis		197	182 (92.4)	15 (7.6)	0 (.0)	.046	379 (96.2)	15 (3.8)	.049

Numbers in parentheses indicate percentages.

the *DTNBP1* gene (P1635: genotype, $\chi^2 = 17.74$, $df = 1$, $p = .000025$; allele $\chi^2 = 17.20$, $df = 1$, $p = .000030$; SNPA: genotype $\chi^2 = 4.63$, $df = 1$, $p = .046$; allele $\chi^2 = 4.51$, $df = 1$, $p = .049$). The minor alleles of P1635 and SNPA, G and T alleles, respectively, were in excess in methamphetamine psychosis when compared with control subjects. To avoid a type I error due to multiple comparison, the Bonferroni correction was applied to the results. The G allele of P1635 was still significantly more frequent in the methamphetamine psychosis patients than in control subjects, but SNPA was not significantly different after correction. P1655 did not show significant differences in distribution of allele and genotype between groups.

Comparison between subgroups of the patients according to clinical phenotypes showed a significant difference in allelic and genotypic distribution of P1635 between the two subgroups divided by duration of psychotic state after therapy, transient and prolonged types (Table 2). The frequency of the minor allele G of P1635 was only 0.8% in control subjects, whereas it was 3.3% in patients with transient psychosis and 8.5% in patients with prolonged psychosis ($p = .027$, compared with transient psychosis). After Bonferroni correction, this was not significant. The other clinical phenotypes, psychosis latency and spontaneous relapse, were not associated with any SNP examined.

Estimation of the pairwise LD between the three SNPs of the *DTNBP1* gene using the D′ and r^2 values as an index showed that P1655, P1635, and SNPA have strong LD (D′ ranging between 0.65 and 1.0) with each other (Table 3). We then analyzed the three-marker haplotypes (Table 4) and found significant differences in patients and control subjects at P1655-P1635-SNPA ($\chi^2 = 27.8$, $df = 6$, global permutation $p = .0005$).

Table 2. Association of the *DTNBP1* Gene with Subgroups of Patients Divided by Clinical Phenotypes

		Genotype				Allele		
	N	C/C	C/G	G/G	*p*	C	G	*p*
P1655								
Latency to Onset of Psychosis, <3Y	96	35 (36.5)	50 (52.1)	11 (11.4)		120 (62.5)	72 (37.5)	
Latency to Onset of Psychosis, ≥3Y	79	36 (45.6)	37 (46.8)	6 (7.6)	.41	109 (69.0)	49 (31.0)	.20
Transient MAP Psychosis	103	44 (42.7)	50 (48.6)	9 (8.7)		138 (67.0)	68 (33.0)	
Prolonged MAP Psychosis	79	29 (36.7)	42 (53.2)	8 (10.1)	.71	100 (63.3)	58 (36.7)	.46
Spontaneous Relapse; No	108	41 (38.0)	54 (50.0)	13 (12.0)		136 (63.0)	80 (37.0)	
Spontaneous Relapse; Yes	77	34 (44.1)	38 (49.4)	5 (6.5)	.40	106 (68.8)	48 (31.2)	.24
P1635		A/A	A/G	G/G		A	G	
Latency to Onset of Psychosis, <3Y	99	89 (89.9)	10 (10.1)	0 (.0)		188 (94.9)	10 (5.1)	
Latency to Onset of Psychosis, ≥3Y	81	71 (87.7)	10 (12.3)	0 (.0)	.63	152 (93.8)	10 (6.2)	.64
Transient MAP Psychosis	107	100 (93.5)	7 (6.5)	0 (.0)		207 (96.7)	7 (3.3)	
Prolonged MAP Psychosis	82	68 (82.9)	14 (17.1)	0 (.0)	.022	150 (91.5)	14 (8.5)	.027
Spontaneous Relapse; No	111	98 (88.3)	13 (11.7)	0 (.0)		209 (94.1)	13 (5.9)	
Spontaneous Relapse; Yes	82	73 (89.0)	9 (11.0)	0 (.0)	.87	155 (94.5)	9 (5.5)	.88
SNPA		A/A	A/T	T/T		A	T	
Latency to Onset of Psychosis, <3Y	99	91 (91.9)	8 (8.1)	0 (.0)		190 (96.0)	8 (4.0)	
Latency to Onset of Psychosis, ≥3Y	82	75 (91.5)	7 (8.5)	0 (.0)	.91	157 (95.7)	7 (4.3)	.910
Transient MAP Psychosis	108	102 (94.4)	6 (5.6)	0 (.0)		210 (97.2)	6 (2.8)	
Prolonged MAP Psychosis	82	73 (89.0)	9 (11.0)	0 (.0)	.170	155 (94.5)	9 (5.5)	.18
Spontaneous Relapse; No	110	104 (94.5)	6 (5.5)	0 (.0)		214 (97.3)	6 (2.7)	
Spontaneous Relapse; Yes	82	74 (90.2)	8 (9.8)	0 (.0)	.26	156 (95.1)	8 (4.9)	.27

Number in parentheses indicate percentages.

Table 3. Pairwise Linkage Disequilibrium Between Single Nucleotide Polymorphisms of the *DTNBP1* Gene

	P1655	P1635	SNPA
P1655		.9643	1.0000
P1635	.0128		.6519
SNPA	.0114	.3522	

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

The estimated haplotype frequency of C-A-A of P1655-P1635-SNPA was significantly lower in patients with methamphetamine psychosis than in control subjects (p = .0013). Conversely, the C-G-T haplotype was significantly higher in patients than in control subjects (p = .0012). Permutation p values of these haplotypes remained significant even after Bonferroni correction. Odds ratios were .62 (95% confidence interval [CI] .51–.77) and 14.9 (95% CI 3.5–64.2), respectively, indicating that the C-A-A haplotype protected against development of methamphetamine psychosis. On the other hand, the C-G-T haplotype was a significant risk factor for development of methamphetamine psychosis.

Discussion

We found a significant association between the *DTNBP1* gene and methamphetamine psychosis in individual marker and haplotype-based case–control analyses. The G allele of P1635 was shown to be a risk factors for methamphetamine psychosis. Numakawa *et al.* (9) reported that the G allele of P1635 was a risk factor for schizophrenia in Japanese; other reports have shown that it was also overtransmitted in Irish (1) but not in German schizophrenia (2). We also found that the G allele of P1635 was in excess in a subgroup showing a prolonged psychotic state, indicating that the allele was a risk for a worse prognosis of psychosis or refractoriness to antipsychotic therapy in patients with methamphetamine psychosis. The T allele of SNPA also showed a nominally significant risk for methamphetamine psychosis. Although it did not remain significant after multiple comparison correction, one study of schizophrenia showed that it was a significant risk (9), whereas another did not (13). The most striking findings in our study were that analyses of a haploype constructed by P1655-P1635-SNPA of the *DTNBP1* gene revealed a strong association with methamphetamine psychosis (p = .0005). The C-A-A haplotype was significantly more common in control subjects than patients with methamphetamine psychosis (p = .0013), implying a substantial protective factor given the odds ratio of .62. The protective haplotype found in our study of methamphetamine psychosis was identical with that previously reported in studies of schizophrenia and psychotic bipolar disorders (12,13). This evidence may indicate that the C-A-A haplotype of *DTNBP1* reduces the liability of individuals who suffer from endogenous psychoses or substance abuse to complications of psychotic symptoms such as delusions and hallucinations. Another possibility should be also considered, however; the C-A-A haplotype may be associated with methamphetamine dependence but not methamphetamine psychosis because all the patients examined in our study suffered not only from methamphetamine psychosis but also dependence. Accordingly, these hypotheses should be examined in other psychotic disorders—for example, psychotic depression, organic psychoses, and cocaine paranoia—as well as in other dependence disorders. In contrast, the C-G-T haplotype was a significant risk for development of methamphetamine psychosis. The frequency of the C-G-T haplotype was small at about 3% in methamphetamine psychosis but almost absent in control subjects, resulting in a strong risk and an odds ratio of 14.9. This haplotype was absent in the UK/Irish studies. In these studies, the C-A-T haplotype was a risk for both schizophrenia and psychotic bipolar disorder; however, this haplotype was rare (<1%) in Japanese samples and was not a significant factor for methamphetamine psychosis. In addition, the UK/Irish studies showed the G-G-T haplotype was as rare as 3% in control subjects but completely absent in schizophrenia, indicating a potent protective factor against schizophrenia. Again, this haplotype was absent in our samples. Such inconsistencies between the present study and UK/Irish studies indicate that the influence of genetic variation of *DTNBP1* on susceptibility to psychiatric disorders differs among the three distinct disorders (i.e., methamphetamine psychosis, schizophrenia, and psychotic bipolar disorder), although the protective C-A-A haplotype was common to all of them. In addition, population differences in SNP frequencies may also affect results. For example, the minor allele frequency of SNPA was .02, which was consistent with another Japanese study (9), but UK/Irish samples showed a frequency of .45 (13). The P1655 frequency was .28 in our samples, which was similar to another Japanese sample (.31) but different from Caucasian samples (.47 in Straub's study [1] and .49 in Williams's study [13]).

The relationship between abnormal dysbindin function and methamphetamine psychosis is unclear. The *DTNBP1* gene encodes a 40-Kd coiled-coil-containing protein that binds to β-dystrobrevin to form dystrophin-associated protein complex (DPC), which is found in postsynaptic densities of the brain (20). *DTNBP1*, however, is particularly expressed in certain axon terminals, notably, mossy fiber synaptic terminals in the cerebellum and hippocampus independent of DPC (20). Talbot *et al.* (10) found that patients with schizophrenia displayed a presynaptic DTNBP1 reduction in the hippocampus, and an inversely correlated increase in vesicular glutamate transporter-1 occurred in the same schizophrenia cases, suggesting a relationship between glutamatergic neurotransmission and DTNBP1. Evidence in vitro showed that overexpression of DTNBP1-enhanced glutamine release accompanied by an increase of presynaptic machinery SNAP25 and synapsin 1 and a knockdown of DTNBP1 by siRNA-reduced glutamate release. Reduced expression of DTNBP1 in schizophrenic brains may result in hypofunction of the glutamatergic system in the brain, which has been promising hypothesis for the pathophysiology of schizophrenia (21,22). Based on the clinical similarity between methamphetamine psychosis and schizophrenia, it has been assumed that shared neural mechanisms, not only dopamine systems but also gluta-

Table 4. Haplotype Frequencies of the *DTNBP1* Gene of Control Subjects and Methamphetamine (MAP) Psychosis

Haplotype P1655-P1635-SNPA	Controls Frequency	MAP Psychosis Frequency	Permutation p
C-A-A	.7101	.6046	.0013
G-A-A	.2741	.3315	.076
C-G-T	.0022	.0318	.0012
C-G-A	.0023	.0178	.11
C-A-T	.0073	.0055	.83
G-G-A	0	.0089	.15
G-A-T	.0039	0	.18

Haplotype analysis was performed by the permutation method. The global permutation p value was .0005.

mate systems, may be involved in the two psychotic disorders. Many lines of evidence from experimental studies using behavioral sensitization by repeated psychostimulant treatment, which has been recognized as an animal model of methamphetamine psychosis (18), showed pivotal roles of N-methyl-D-aspartate (NMDA) receptors and glutamate systems in the development of behavioral sensitization. Thus repeated administration of amphetamine or cocaine produces behavioral sensitization with enhanced efflux of glutamate in the ventral tegmental area (VTA) and accumbens, which are key brain structures for sensitization phenomena (23,24). NMDA receptor antagonists, including the noncompetitive antagonist MK-801, prevent behavioral sensitization to amphetamines when administered systemically or microinjected into the VTA (25–28). In contrast, phencyclidine, another NMDA antagonist, exacerbates amphetamine-induced abnormal behaviors and a hyperdopaminergic state in the prefrontal cortex and striatum (29–31). Amphetamines can also directly inhibit the NMDA receptor complex (32). Although the roles of NMDA receptors and glutamatergic systems in animal models of methamphetamine psychosis seem to be complex, our findings may indicate that variants of *DTNBP1* affect susceptibility to methamphetamine psychosis by implication of glutamatergic neurotransmission. In addition, DTNBP1 was shown to enhance phosphorylation of AKT protein by PI3-kinase and protect against neuronal cell death. Impaired PI3-kinase-Akt signaling and a genetic association with the *AKT1* gene were found in schizophrenia (20,33,34). Previously, we also found a significant association of the *AKT1* haplotype with the same patients of methamphetamine psychosis (35). It is possible that DTNBP1 confers susceptibility to methamphetamine psychosis via the PI3-kinase-Akt signaling cascade. In vitro evidence of interaction between dysbindin and dopamine system was recently reported. Kumamoto *et al.* (36) found that mRNA of disbindin expressed in the mouse substantia nigra, that suppression of dysbindin expression in PC 12 cells resulted in an increase of dopamine release, and that overexpression of dysbindin produced a tendency to decrease dopamine release. This finding suggests that dysbindin dysfunction may induce susceptibility to methamphetamine psychosis through interaction with dopamine systems.

Alternatively, the effect of DTNBP1 on cognitive ability should be considered. In an analysis of the phenotype–haplotype relationship, Williams *et al.* (13) found that the C-A-A protective haplotype was significantly associated only with higher educational attainment. A longitudinal study of childhood and adolescent antecedents of drug and alcohol problems in adulthood showed that, for both males and females, educational attainment was directly associated with a reduced risk for substance use problems (37). In this respect, higher educational attainment due to carrying the C-A-A haplotype might be involved in a reduced risk for methamphetamine psychosis, and the phenotype of higher educational attainment might be a common protective factor in methamphetamine psychosis and schizophrenia. Further studies are required to confirm this possibility.

Although our results remained significant after Bonferroni correction, it is possible that this was a chance finding resulting from reduced power due to small sample size. Analysis showed, however, that our sample size for the three SNPs had powers of .9994, 1.0000, and .9594 to detect an effect size (w = .1892, .5388, and .1263, respectively), with a significance level of .05 to detect significant associations in allelic analysis between control subjects and subjects with methamphetamine psychosis. Our total sample size is therefore large enough statistically, and it is unlikely that our positive findings results from reduced power. When methamphetamine psychosis patients are divided into subgroups according to clinical phenotypes, however, the statistical power may be reduced. It is possible that a rare haplotype C-G-T as a risk for methamphetamine psychosis may result from a chance fluctuation. In addition, a false-positive association owing to population stratification could not be excluded in this study despite careful matching of control subjects and patients. Our findings should be confirmed in larger samples and in different populations.

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