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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shuffling gait. Because these side effects were extremely disturbing, aripiprazole was ultimately discontinued and substituted with risperidone 2 mg daily. Persecutory delusions attenuated significantly within five days, and parkinsonism gradually resolved after discontinuing aripiprazole.

Aripiprazole acts as a partial agonist at the D_2 and 5-HT_{1A} receptors and is also an antagonist at the 5-HT_{2A} receptor. The pharmacodynamics of aripiprazole is thought to be effective and well tolerated for schizophrenic patients [4]. However, several cases of aripiprazole-associated EPS have been reported [2,5]. For example, Cohen et al. [1] reported on two cases of depressive patients presenting with marked EPS, associated with concomitant use of aripiprazole and antidepressants (sertraline and venlafaxine). It was postulated that serotonergic agents with modest dopaminergic activity (e.g., sertraline) or noradrenergic activity (e.g., venlafaxine) might have modified aripiprazole's neurochemical effects.

In the case of Mr. A—a new-onset, drug-naïve schizophrenic patient—aripiprazole was the only agent prescribed. This medication, although partially effective, exerted intolerable EPS. Although it was possible that our patient's EPS were related to his genetic propensity for parkinsonism, our report suggests that a systemic investigation into aripiprazole's pharmacodynamic and side-effect profile would be welcome. We also recommend slow titration to target doses among some high-risk patients (e.g., young males) to decrease the risk of EPS and akathisia with aripiprazole in clinical settings.

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Successful Treatment of Severe Antidepressant-Induced Nausea with a Combination of Milnacipran and Olanzapine

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Nausea is one of the most problematic side effects induced by newer antidepressants, including selective serotonin reuptake inhibitors (SSRIs) and serotonin noradrenaline reuptake inhibitors (SNRIs). We report on a patient with obsessive-compulsive disorder and comorbid major depressive disorder who experienced severe nausea induced by SSRIs and an SNRI. She was successfully treated with a combination of milnacipran and olanzapine without nausea.

A 44-year-old female patient presented with the behavior of compulsively videotaping of entire TV programs on every channel. She recognized that this behavior was absurd and unnecessary, but its cessation made her anxious and irritable. When she could not record the TV programs herself, she asked her relatives to videotape them. When her relatives were not available, she asked video-recording companies to videotape the programs. Her room was filled with videotapes. She consulted a psychiatrist and was administered paroxetine. She could not continue this therapy, because a single paroxetine dose of 10 mg induced severe nausea. She visited several psychiatrists and was administered paroxetine, fluvoxamine, or milnacipran, with sulpiride as an antiemetic. However, the concomitant use of sulpiride could not suppress the severe nausea induced by antidepressants; a single dose of the smallest tablet resulted in intolerable nausea. After a psychiatrist told her that there was nothing to do for an obsessive-compulsive patient extremely sensitive to antidepressant-induced nausea, depressive symptoms appeared and gradually increased in severity. Finally, she was only able to videotape TV programs and spent most of the day in bed because of lassitude and loss of motivation. She visited our hospital on the advice of her relatives. She was administered a very low dose of milnacipran, because the incidence of nausea with milnacipran has been reported to be lower than that of SSRIs [5]. She was advised to buy a pill cutter and to try 1/8 of a 15-mg tablet once daily. This single low dose of milnacipran did not induce nausea. The number of doses per day and the amount of the dosage were gradually increased. She tolerated a dosage of 3.75 mg four times daily, but further increases in the daily dosage induced nausea. Because SSRI-induced nausea has been reported to be mediated via 5-HT_3 receptors [2], the concomitant administration of a drug with a 5-HT_3-inhibiting effect seemed to be appropriate. Olanzapine, an antipsychotic drug with a 5-HT_3-inhibiting effect, has been reported to be effective for obsessive-compulsive disorder in combination with SSRIs [1]. Therefore, a single dosage of 2.5 mg was added to the milnacipran treatment. After the administration of olanzapine, the daily dosage of milnacipran

could be gradually increased. When the daily dosage of milnacipran reached 90 mg/day, her depressive symptoms began to ameliorate. It became possible for her to do household chores and go outside for shopping. At that time, however, her excessive videotaping remained unchanged. When the daily dosage of milnacipran reached 150 mg/day, her excessive videotaping began to ameliorate. It became possible for her to cease videotaping new serial dramas. Currently, she is taking milnacipran 200 mg/day and olanzapine 2.5 mg/day. No apparent side effects have emerged. Her consumption of videotapes has become less than 10% of that at the first visit to our hospital.

Individual differences in pharmacokinetics and pharmacodynamics, mediated mainly via genetic polymorphisms, can lead to individual differences in susceptibility to nausea and other side effects induced by antidepressants as well as the antidepressant effect itself [4]. This case suggests the way that patients who are extremely sensitive to antidepressant-induced nausea should be treated. First, the initiation of antidepressants at a very low dosage should be considered. Intolerance of a single dose of the smallest tablet does not necessarily mean complete intolerance of an antidepressant. Second, the concomitant use of a 5-HT_3-blocking agent should be considered. Dopamine antagonists, such as sulpiride and metoclopramide, seem to be frequently used as antiemetics with antidepressants in Japan. However, their antidopaminergic effect is indirect for antidepressant-induced nausea. If the use of olanzapine is undesirable, mianserin, a tetracyclic antidepressant may be useful as a 5-HT_3 inhibitor [3].

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Rebound of Weight Gain Following Topiramate Cessation

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Glutamatergic system plays an important role in compulsive and/or addictive behaviors [5], which are associated with environmental cues [1]. Topiramate notably blocks the AMPA/kainate subtype of glutamate receptors [4]. Diverse studies and case reports have recently raised the interest of topiramate in the treatment of addictive disorders [6], obesity, and weight gain associated with antipsychotic drugs [2]. Despite this promising treatment for obesity [3], duration of treatment and its modality cessation remain understudied till to date.

We report here a case of weight rebound following topiramate cessation. Mr. A, a 28-year-old, Caucasian, consulted for the treatment of obesity (BMI = 32). He reported having received previously multiple dietary treatments, which was followed by weight gain and one unsuccessful orlistat treatment. Assessment of eating behaviors revealed no binge eating. The subject has 2 daily meals with 4–6 snacks equivalent to over half of daily caloric intake. Snacks were related to specific cues (such as specific food stimuli: sweets or chocolates and "small" sandwiches and chips...). He received four sessions in order to explore specific cues and determine their role in his obesity. When topiramate was started at a dose of 25 mg/day, he was advised to eat as usual, however, to observe his snacking and reducing it to twice daily as one of the treatment goals. Topiramate was then increased weekly by 25 mg, and by 50 mg/week from week 4 onwards, until changes were observed in eating behavior (snacking reduction ≥ 50%) at a dosage of 125 mg/day. Mr. A reduced food consumption within week 5, leading to a reduction in "snacking" to twice daily. He felt less attracted by food cues finding it easier to resist the temptation to snack, which was maintained at 1 to 2 times/day representing less than 1/4 of total daily caloric intake. He lost 5 kg by week 9. He then remained with that stable weight for a month. Topiramate dosage was increased to 175 mg/day. He experienced distressing, word-finding difficulties as well as 'mental block' sensations without further weight loss which led him to stop the treatment abruptly. Consequently, the distressing side effects disappeared within two days.

After 6 weeks, he consulted again describing a rebound of snacking (6–8/day) within the first week of topiramate cessation associated with weight gain (8.5 kg) and highest peak in body weight history. He then refused topiramate, afraid of a subsequent weight rebound and distressing side effects at higher dosages. A Cognitive Behavior Therapy (CBT) was then started leading to a moderate improvement during the four following months (3–4 snacks daily, weight loss 3.5 kg).

One can hypothesize that the observed weight rebound phenomenon could be prevented by more prolonged treatment, progressive discontinuation or adjunctive CBT. Topiramate discontinuation strategies should be studied. Furthermore, the addition of CBT in deconditioning the cues brought forth by topiramate and their

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The G196A polymorphism of the brain-derived neurotrophic factor gene and the antidepressant effect of milnacipran and fluvoxamine

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Abstract

Prediction of the response to different classes of antidepressants has been an important matter of concern in the field of psychopharmacology. The purpose of the present study was to investigate whether the G196A polymorphism of the brain-derived neurotrophic factor (BDNF) gene is associated with the antidepressant effect of milnacipran, a serotonin norepinephrine reuptake inhibitor, and fluvoxamine, a selective serotonin reuptake inhibitor. The subjects of our previous study of milnacipran ($n = 80$) and fluvoxamine ($n = 54$) were included in the present study. Severity of depression was assessed with the Montgomery Åsberg depression rating scale (MADRS). Assessments were carried out at baseline and at 1, 2, 4 and 6 weeks of treatment. Polymerase chain reaction was used to determine allelic variants. In all subjects receiving milnacipran or fluvoxamine, the *G/A* genotype of the BDNF G196A polymorphism was associated with a significantly better therapeutic effect in the MADRS scores during this study. When milnacipran and fluvoxamine-treated subjects were analysed independently, the *G/A* genotype group showed greater reduction of MADRS scores than other genotype groups, irrespective of which antidepressant was administered. These results suggest that the BDNF G196A polymorphism in part determines the antidepressant effect of both milnacipran and fluvoxamine.

Keywords

antidepressant effect, genetic polymorphism, fluvoxamine, major depressive disorder, milnacipran

Introduction

Prediction of the response to different classes of antidepressants has been an important matter of concern in the field of psychopharmacology. A consistent relationship between the antidepressant effect and the plasma concentrations of selective serotonin (5-HT) reuptake inhibitors (SSRIs) has not been obtained (Burke and Preskorn, 1999), although early pharmacokinetic studies identified significant relationships between the antidepressant effect and plasma concentrations of several tricyclic antidepressants (Perry *et al.*, 1987). In terms of serotonin norepinephrine (NE) reuptake inhibitors (SNRIs), venlafaxine showed a positive association between antidepressant efficacy and plasma concentrations (Charlier *et al.*, 2002), while this relationship was not observed for milnacipran (Higuchi *et al.*, 2003).

Recent progress in pharmacogenetics has facilitated investigation of the relationship between genetic polymorphisms and the antidepressant response. Genetic polymorphisms of the 5-HT and NE transporter have been investigated intensively, because they are believed to be the primary target of SSRIs and SNRIs. As a result,

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several interesting findings have been reported (Malhotra *et al.*, 2004; Yoshida *et al.*, 2004), but there is no consistent evidence to predict the antidepressant response. Thus, further pharmacogenetic studies of antidepressants must be performed in order to predict the antidepressant response adequately.

Recently, it has been proposed that antidepressants eventually cause critical genes to be activated or inactivated, no matter how they act on receptors and enzymes (Stahl, 2000). One of the likeliest candidate genes is brain-derived neurotrophic factor (BDNF), which belongs to a family of neurotrophic factors including neurotrophin-3/4/5 and nerve growth factor and has an important role as a potent modulator of synaptic transmission and plasticity. Substantial evidence supports that BDNF is involved not only in cognitive processes, such as memory and learning, but also in the pathophysiology of mood disorders and in the mechanism of antidepressant action, as follows. Expression of BDNF mRNA is down-regulated by either acute or repeated stressful conditions of immobilization (Smith *et al.*, 1995). An antidepressant effect in both the learned helplessness and the forced swimming tests is observed as early as 3 days after a single infusion of BDNF into the hippocampus (Shirayama *et al.*, 2002). Chronic treatment with tranylcypromine, a monoamine oxidase inhibitor, caused a significant increase in BDNF mRNA in the rat hippocampus (Russo-Neustadt *et al.*, 1999), and chronic administration of amitriptyline, a tricyclic antidepressant, significantly increased BDNF protein levels in the rat hippocampus and prefrontal cortex (Okamoto *et al.*, 2003). Thus, the BDNF gene is a plausible candidate gene for mood disorders and pharmacogenetic studies of the antidepressant response.

The G196A polymorphism in exon IIIA is located within the propeptide region of the BDNF gene. Several association studies have examined the G196A polymorphism and vulnerability for bipolar or major depressive disorders (Hong *et al.*, 2003; Nakata *et al.*, 2003). These studies have found no major role for the polymorphism in the pathophysiology of mood disorders, although Egan *et al.* (2003) reported that it influences human memory and hippocampal function. So far only one pharmacogenetic study of antidepressants and the BDNF G196A polymorphism has been carried out (Tsai *et al.*, 2003); in this study, the response to treatment with fluoxetine was evaluated for only 4 weeks and the response rate was as low as 33.6%.

In the current 6-week study, we examined the effect of the BDNF G196A polymorphism on the antidepressant effect of milnacipran, an SNRI, and fluvoxamine, an SSRI. In addition, we investigated another polymorphism of C132T in the non-coding region of exon V of the BDNF gene, which was detected and named C270T by Kunugi *et al.* (2001). Plasma concentrations of milnacipran and fluvoxamine were investigated to evaluate patients' compliance and an influence on the antidepressant effect.

Materials and methods

Subjects

The subjects in our previous studies (Yoshida *et al.*, 2002; Yoshida *et al.*, 2004) were included in the present study. The subjects were Japanese patients who fulfilled DSM-IV criteria for a diagnosis of major depressive disorder and whose scores on the Montgomery Åsberg depression rating scale (MADRS) (Montgomery and Asberg, 1979) were 21 or higher. Patients with other axis I disorders (including dementia, substance abuse, dysthymia, panic disorder, obsessive–compulsive disorder and generalized anxiety disorder) and those with axis II disorders determined by clinical interview were excluded. Patients with a history of childhood disorders were also excluded, as were patients with severe non-psychiatric medical disorders. The patients were 20–69 years of age and had been free of psychotropic drugs at least 14 days before entry into the study. After complete description of the study to the subjects, written informed consent was obtained. This study was approved by the Ethical Committee of Akita University School of Medicine. The clinical characteristics of the patients are shown in Table 1. There was no significant difference between responders and non-responders in regard to sex, age, number of previous episodes and presence of melancholia. There was no significant difference in clinical characteristics when milnacipran and fluvoxamine-treated patients were analysed independently (data not shown). The number of previous depressive episodes was very low. Indeed, most of the patients (milnacipran: 64/80, fluvoxamine: 41/54) were in their first episode.

Milnacipran treatment

Milnacipran was administered twice daily (the same dose after dinner and at bedtime) for 6 weeks. The initial total daily dose was 50 mg/day, and after a week it was increased to 100 mg/day. Patients with insomnia were prescribed brotizolam, 0.25 or 0.5 mg, a benzodiazepine sedative hypnotic, at bedtime. No other

Table 1 Clinical characteristics of the patients in the milnacipran and fluvoxamine treatment (responders and non-responders)

	Responders ($n = 85$)	Nonresponders ($n = 49$)		p
Sex (male/female)	34/51	16/33	$\chi^2 = 0.72$	0.40[a]
Age (years) (± SD)	50.7 ± 12.4	52.2 ± 12.8	$t = -0.68$	0.50[b]
Number of previous episodes (± SD)	0.48 ± 1.7	0.33 ± 0.7	$t = 0.77$	0.44[b]
Melancholia (+/-)	21/64	15/34	$\chi^2 = 0.55$	0.46[a]

[a] Analysis performed with the use of the χ^2 test.

[b] Analysis performed with the use of the unpaired t test.

psychotropic drugs were permitted during the study. Of 96 enrolled patients, ten did not complete the study; five patients because of side effects, one patient because of severe insomnia and four patients without explanation. Of the 86 patients who completed the 6-week study, six patients were excluded from the current analysis because plasma samples revealed very low milnacipran concentrations, indicative of poor compliance. Patients who completed the study included 52 women and 28 men, 49 outpatients and 31 inpatients, and ranged from 25 to 69 years of age (mean age = 51.4 ± 12.2 (±SD)).

Fluvoxamine treatment

Fluvoxamine was administered twice daily (the same dose after dinner and at bedtime) for 6 weeks. The initial total daily dose was 50 mg/day. The daily dose was increased to 100 mg/day after a week and was increased to 200 mg/day after another week. Concomitant administration of psychotropic drugs was restricted as in the milnacipran study. Of 66 enrolled patients, nine did not complete the study; four patients because of side effects and five patients without explanation. Of the 57 patients who completed the 6-week study, three patients were excluded from the current analysis because plasma samples revealed very low fluvoxamine concentrations, indicative of poor compliance. Patients who completed the study included 32 women and 22 men, 43 outpatients and 11 inpatients, and ranged from 24 to 69 years of age (mean age = 51.2 ± 13.2 (±SD)).

Data collection

Depression symptom severity was assessed with the use of the MADRS. Assessments were conducted at baseline and at 1, 2, 4 and 6 weeks after initiation of antidepressant treatment. A clinical response was defined as a 50% or greater decrease in the baseline MADRS score. Clinical remission was defined as a final MADRS score less than ten (Hawley *et al.*, 2002). Collection of blood samples was performed 12 hours after drug administration at bedtime, 4 weeks after initiation of each antidepressant treatment.

Genotyping

The BDNF G196A polymorphism was determined by a minor modification of the method of Tsai *et al.* (2003). The BDNF C132T polymorphism was determined by a minor modification of the method of Szekeres *et al.* (2003). Primers and enzymes used in this study were the same as previous studies; the conditions of the polymerase chain reaction and the chemical reagents were adjusted to our instruments.

Quantification of plasma milnacipran/fluvoxamine concentration

Plasma concentrations of milnacipran were measured with high performance liquid chromatography (HPLC). Details of the method have been described previously (Higuchi *et al.*, 2003). Plasma concentrations of fluvoxamine were measured with HPLC. Details of the method have been described previously (Ohkubo *et al.*, 2003). Genotyping and measurement of plasma concentrations were performed by laboratory personnel blind to the identity and clinical antidepressant effect of the patients. Moreover, clinicians were unaware of the genotyping results and the plasma milnacipran concentrations of each patient.

Statistical analysis

Differences in patient characteristics were analysed with the use of the unpaired t-test or chi-square test where appropriate. Differences in the MADRS scores during this study were examined with the use of two-way repeated measures analysis of variance (ANOVA), with genotype and time as factors. When significant interaction between factors was observed, contrasts were used to enable comparisons between each two of the three genotype groups. Differences in the MADRS scores at each evaluation point were examined with the one-way factorial ANOVA followed by the Fisher's PLSD test. Genotype deviation from Hardy-Weinberg equilibrium was evaluated by the chi-square test. Genotype distribution and allele frequencies were analysed with the use of the chi-square test. Plasma concentrations of milnacipran or fluvoxamine were analysed with the use of one-way factorial ANOVA in each genotype group; an unpaired t-test was then used to analyse differences between groups who were or were not responsive to milnacipran or fluvoxamine. Statistical analysis was performed using StatView version 5.0 (SAS Institute Inc., Cary, NC), except the two-way repeated measures ANOVA with contrasts was performed using SuperANOVA version 1.11 (Abacus Concepts, Inc., Berkeley, CA). Power analysis was performed with the use of G·Power (Buchner *et al.*, 1996). All tests were two-tailed; alpha was set at 0.05.

Results

Minor allele frequencies for the C132T polymorphism were very low and similar to those reported by Kunugi *et al.* (2001) and Szekeres *et al.* (2003); 5.0% in the patients treated with milnacipran and 3.7% in those treated with fluvoxamine. Therefore, only the G196A polymorphism was included in the statistical analysis. The observed genotype frequencies of the G196A polymorphism were within the distribution expected according to the Hardy-Weinberg equilibrium. As the authors reported that response to fluvoxamine was associated with allelic variations of the 5-hydroxytriptamine transporter gene-linked polymorphic region (5-HTTLPR) (Yoshida *et al.*, 2002) and response to milnacipran was associated with those of the norepinephrine transporter T-182C and G1287A polymorphisms (Yoshida *et al.*, 2004), it was necessary to confirm these polymorphisms to be controlled. The genotype distribution of these genotypes was not significantly different among the *G/G*, *G/A* and *A/A* genotype groups of the BDNF G196A polymorphism (data not shown).

Fig. 1 shows the MADRS scores over time in relation to the BDNF G196A polymorphism for all subjects receiving fluvoxamine or milnacipran. There was no significant difference in baseline MADRS scores among each genotype group. Two-way repeated

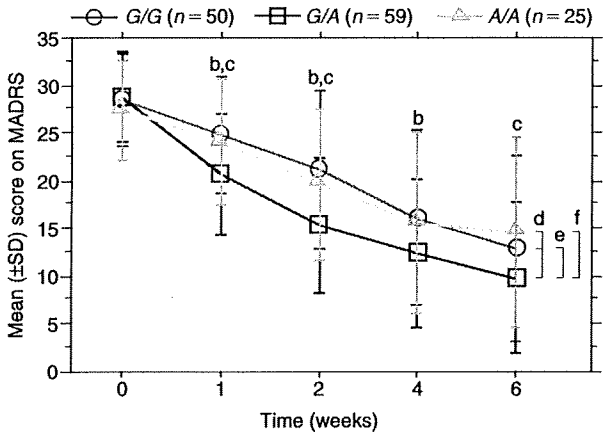

Figure 1 MADRS scores during 6 weeks of milnacipran/fluvoxamine treatment in three BDNF G196A genotype groups[a]

[a] Each point represents te mean score ± SD. Differences in the MADRS scores during this study were examined with the use of two-way repeated measures ANOVA with contrasts. Differences in the MADRS scores at each evaluation point were examined with the use of one-way factorial ANOVA followed by Fisher's PLSD test.

[b] Significant differences at each point between the *G/A* and *G/G* groups ($p = 0.0009$ at week 1, $p = 0.0001$ at week 2 and $p = 0.025$ at week 4).

[c] Significant difference at each point between the *G/A* and *A/A* groups ($p = 0.032$ at week 1, p = 0.019 at week 2 and 0.029 at week 6).

[d] Significant geneotype × time interaction among all three genotype groups (F = 3.64, df = 8, $p = 0.0004$).

[e] Signicant genotype × time interaction between the *G/A* and *G/G* groups (F = 5.21, df = 4, $p = 0.0004$)

[f] Significant genotype × time interaction between the *G/A/* and *A/A*: groups (F = 3.99, df = 4, $p = 0.0034$).

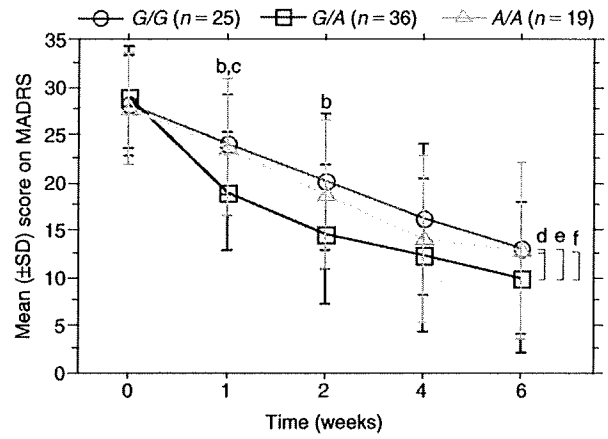

Figure 2 MADRS scores during 6 weeks of milnacipran treatment in three BDNF G196A genotype groups[a]

[a] Each point represents the mean score ± SD. Differences in the MADRS scores during this study were examined with the use of two-way repeated-measures ANOVA with contrasts. Differences in the MADRS scores at each evaluation point were examined with the use of one-way factorial ANOVA followed by Fisher's PLSD test.

[b] Significant difference at each point between the *G/A* and *G/G* groups ($p = 0.0031$ at week 1 and $p = 0.0056$ at week 2).

[c] Significant difference between the *G/A* and *A/A* groups (p=0.011 at week 1).

[d] Significant genotype × time interaction among all three genotype groups (F = 2.30, df = 8, p = 0.021).

[e] Significant genotype × time interaction between the *G/A* and *G/G* groups (F = 3.54, df = 4, $p = 0.0077$).

[f] Significant genotype x time interaction between the *G/A* and *A/A* groups (F = 2.56, df = 4, $p = 0.039$).

measures ANOVA including all three genotype groups indicated a significant genotype × time interaction. Contrast analysis indicated a significant genotype × time interaction between the *G/A* and *G/G* genotype groups. The MADRS score of the *G/A* genotype group was significantly lower than that of the G/G genotype group at 1, 2 and 4 weeks. Contrast analysis indicated a significant genotype x time interaction between the *G/A* and *A/A* groups. The MADRS score of the *G/A* genotype group was significantly lower than that of the *A/A* group at 1, 2 and 6 weeks. Contrast analysis indicated no significant genotype × time interaction between the *G/G* and *A/A* genotype groups (F = 0.99, df = 4, $p = 0.41$). There was no significant difference in the MADRS score at any evaluation point between the *G/G* and *A/A* genotype groups. When milnacipran- and fluvoxamine-treated subjects were analysed independently, the *G/A* genotype group showed greater reduction of MADRS scores than other genotype groups, irrespective of which antidepressant was administered (Figs. 2 and 3). Mean plasma concentrations of milnacipran were 92.3 ± 50.4 (±SD) ng/ml, 88.1 ± 31.1 ng/ml and 91.7 ± 36.2 ng/ml for the *G/G*, *G/A* and *A/A* genotype groups, respectively. There was no significant difference among the groups (F = 0.99, df = 2, 77, $p = 0.90$). Mean plasma concentrations of fluvoxamine were 169.1 ± 174.7 (±SD) ng/ml, 155.1 ± 118.6 ng/ml and 94.8 ± 35.3 ng/ml for the *G/G*, *G/A* and *A/A* genotype groups respectively. There was no significant difference among the groups (F = 0.65, df = 2, 51, $p = 0.53$).

Table 2 shows the genotype distribution and allele frequencies of responders and non-responders for all subjects receiving milnacipran or fluvoxamine. The proportion of responders was higher in *G/A* subjects than in subjects of other genotypes, but it did not reach a significant difference. There was no significant difference in the allele frequencies between responders and non-responders. The proportion of responders was non-significantly higher in *G/A* subjects than in subjects of other genotypes, irrespective of which antidepressants were administered (Table 3 and Table 4). When remitters and non-responders were compared, there was also no significant difference in the genotype distribution ($\chi^2 = 2.53$,

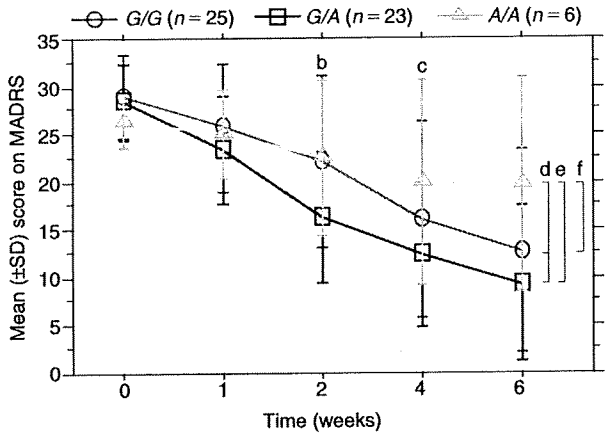

Figure 3 MADRS scores during 6 weeks of fluvoxamine treatment in three BDNF G196A genotype groups[a]

[a] Each point represents the mean score ± SD. Differences in the MADRS scores during this study were examined with the use of two-way repeated-measures ANOVA with contrasts. Differences in the MADRS scores at each evaluation point were examined with the use of one-way factorial ANOVA followed by Fisher's PLSD test.
[b] Significant difference between the *G/A* and *G/G* groups ($p = 0.015$ at week 2).
[c] Significant difference between the *G/A* and *A/A* groups ($p = 0.024$ at week 6).
[d] Significant genotype × time interaction among all three genotype groups ($F = 2.83$, $df = 8$, $p = 0.0053$).
[e] Significant genotype × time interaction between the *G/A* and *A/A* groups ($F = 4.55$, $df = 4$, $p = 0.0015$).
[f] Significant genotype × time interaction between the *G/G* and *A/A* groups ($F = 2.77$, $df = 4$, $p = 0.029$).

$df = 2$, $p = 0.12$ for the milnacipran treatment, $\chi^2 = 3.25$, $df = 2$, $p = 0.20$ for the fluvoxamine treatment and $\chi^2 = 4.26$, $df = 2$, $p = 0.12$ for both treatments) and genotype frequencies ($\chi^2 = 2.53$, $df = 1$, $p = 0.52$ for the milnacipran treatment, $\chi^2 = 0.64$, $df = 1$, $p = 0.64$ for the fluvoxamine treatment and $\chi^2 = 4.26$, $df = 1$, $p = 0.63$ for both treatments) (data not shown).

The plasma concentrations of milnacipran or fluvoxamine were not significantly different between responders and nonresponders, as shown in our previous studies (Yoshida *et al.*, 2002; Yoshida *et al.*, 2004).

This study of both milnacipran and fluvoxamine had a power of 0.16 to detect a small effect, 0.88 to detect a medium effect and 0.99 to detect a large effect in the genotype distribution ($n = 134$). For the allele frequency analysis ($n = 268$), this study had a power of 0.37 to detect a small effect, 0.99 to detect a medium effect and 0.99 to detect a large effect. In the power analysis, effect size conventions were determined according to the method of Buchner *et al.* (1996) as follows: small effect size = 0.10, medium effect size = 0.30 and large effect size = 0.50 (alpha = 0.05).

Discussion

The present study revealed that the BDNF G196A polymorphism affected the efficacy of both milnacipran and fluvoxamine. The *G/A* genotype of this polymorphism was associated with a significantly better therapeutic effect in the MADRS scores during this study, although the difference in final therapeutic response was not significant between the *G/A* and other genotype groups.

The results of this study are not well explained by the findings by Egan *et al.* (2003). Their human study showed that the *A* allele was associated with poorer episodic memory, abnormal hippocampal activation as determined by functional magnetic resonance imaging (fMRI), and lower hippocampal n-acetyl aspartate levels as assayed by MRI spectroscopy. According to their expression study, high concentrations of KCl induced detectable release of *G*-BDNF, whereas the activity-dependent release of *A*-BDNF was severely reduced and sometimes not detectable. Thus, the presence of the *G* allele is related to appropriate hippocampal function, neuronal function and activity-dependent BDNF release. In consideration of these findings, it is difficult to interpret the present results.

However, several aspects should be considered before trying to interpret our study based on the findings by Egan *et al.* (2003). First, the behavioural and mood abnormalities associated with major depressive disorder appear to result from disturbances mainly in the temporolimbic-frontal-caudate network (Drevets, 1999; Czeh *et al.*, 2001), although several lines of research support the notion that the hippocampus is also an important

Table 2 Genotype distribution and allele frequencies in responders and non-responders (milnacipran/fluvoxamine treatment)[a]

	Genotype distribution[b]			Allele frequency[c]	
	G/G	*G/A*	*A/A*	*G*	*A*
Responder	29 (34.1%)	43 (50.6%)	13 (15.3%)	101 (59.4%)	69 (40.6%)
Non-responder	21 (42.9%)	16 (32.6%)	12 (24.5%)	58 (59.2%)	40 (40.8%)

[a] Analysis performed with the use of the χ^2 test.
[b] No significant difference between responders and nonresponders ($\chi^2 = 1.32$, $df = 2$, $p = 0.12$).
[c] No significant difference between responders and nonresponders ($\chi^2 = 0.001$, $df = 1$, $p = 0.97$).

Table 3 Genotype distribution and allele frequencies in responders and non-responders (milnacipran treatment)[a]

	Genotype distribution[b]			Allele frequency[c]	
	G/G	*G/A*	*A/A*	*G*	*A*
Responder	13 (26.0%)	26 (52.0%)	11 (22.0%)	52 (52.0%)	48 (48.0%)
Non-responder	12 (40.0%)	10 (33.3%)	8 (26.7%)	34 (56.7%)	26 (43.3%)

[a] Analysis performed with the use of the χ^2 test.
[b] No significant difference between responders and non-responders ($\chi^2 = 2.80$, df = 2, $p = 0.25$).
[c] No significant difference between responders and non-responders ($\chi^2 = 0.030$, df = 1, $p = 0.57$).

Table 4 Genotype distribution and allele frequencies in responders and non-responders (fluvoxamine treatment)[a]

	Genotype distribution[b]			Allele frequency[c]	
	G/G	*G/A*	*A/A*	*G*	*A*
Responder	16 (45.7%)	17 (48.6%)	2 (5.7%)	49 (70.0%)	21 (30.0%)
Non-responder	9 (47.4%)	6 (31.6%)	4 (21.0%)	24 (63.2%)	14 (36.8%)

[a] Analysis performed with the use of the χ^2 test.
[b] No significant difference between responders and nonresponders ($\chi^2 = 3.45$, df = 2, $p = 0.18$).
[c] No significant difference between responders and nonresponders ($\chi^2 = 0.53$, df = 1, $p = 0.47$).

region in the pathophysiology of major depressive disorder (Campbell and Macqueen, 2004). Therefore, the functional effect of the BDNF G196A polymorphism on the temporolimbic-frontal-caudate network is necessary to understand the present results adequately. However, such information is extremely limited; to our knowledge, there have been no reports investigating this issue using functional brain imaging, such as fMRI or positron emission tomography.

Only one cognitive study (Foltynie *et al.*, 2005) investigated the effect of the BDNF G196A polymorphism on performance of planning ability in Parkinson's disease using the Tower of London (TOL) task, a test of working memory (Robbins, 1996). The TOL task is reported to increase relative regional cerebral blood flow in the dorsolateral prefrontal cortex, lateral premotor cortex, rostral anterior cingulate cortex and dorsal caudate nucleus (Dagher *et al.*, 1999). Foltynie *et al.* (2005) revealed that the *A* allele of the BDNF G196A polymorphism was associated with better performance at the TOL task. This result is inconsistent with the results by Egan *et al.* (2003), who reported that the presence of the *A* allele was associated with impaired function in the hippocampus. The exact mechanism underlying this discrepancy is unclear. The study by Foltynie *et al.* (2005) was performed in Parkinson's disease not in major depressive disorder. However, it is possible that the functional effects of the BDNF G196A polymorphism differ among areas of the brain in major depressive disorder, and this regional difference in the temporolimbic-frontal-caudate network and the hippocampus may contribute to the better antidepressant effect in patients with the *G/A* genotype.

Additionally, some other studies indicated that subjects heterozygous for the BDNF G196A polymorphism have significant differences in expression of dichotomous or quantitative phenotypes than those homozygous for either allele. Momose *et al.* (2002) reported that homozygosity of the BDNF G196A polymorphism was more frequent in patients with Parkinson's disease. This finding suggests that the *G/A* genotype is less susceptible to Parkinson's disease than other genotypes. Tsai *et al.* (2003) reported a trend to a higher percentage change of the total Hamilton Depression Rating score for heterozygote patients in comparison to homozygote patients after fluoxetine treatment for 4 weeks. Their results are consistent with those of the present study and suggest that the *G/A* genotype is related to a favourable antidepressant effect. Besides the possible regionally different effects of the BDNF G196A polymorphism on brain function, another possibility is that the polymorphism may be in linkage disequilibrium with an as yet unidentified functional polymorphism with a molecular heterotic effect (Comings and MacMurray, 2000).

One major limitation of this study is the relatively small number of subjects, especially in the fluvoxamine arm. A second limitation is the relatively small end point treatment differences. These limitations may increase the possibility of a false positive and make it difficult to conclude that the BDNF G196A polymorphism is the common genetic factor for prediction of the antidepressant effect of both milnacipran and fluvoxamine. Further studies with a larger number of subjects are needed not only to confirm the results of this study but also to investigate the interaction of many genes, including the BDNF gene, on the mechanisms of antidepressant action.

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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-1	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

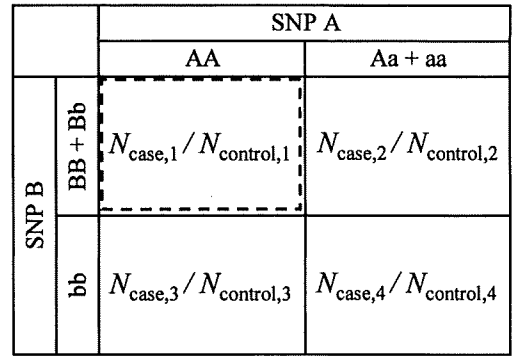

FIG. 1. Rule table using combination of two single nucleotide polymorphisms (SNPs). $N_{case,i}$ and $N_{control,i}$ represent the numbers of case and control subjects, respectively, belonging to rule no. i. The rule circled by a dotted line denotes subjects with the genotype AA of SNP A and the B allele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

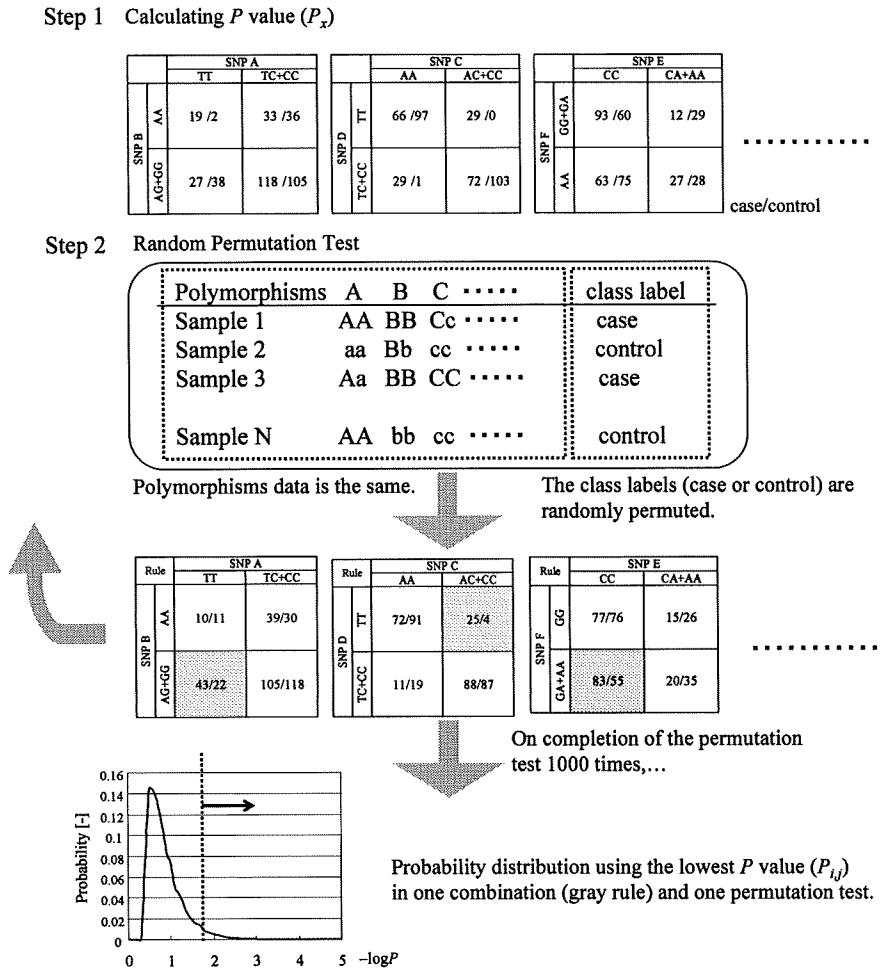

FIG. 2. Selection procedure for risk factor candidates (RFCs) using binomial and random permutation test (BRP) in combination of two polymorphisms. The analysis procedure has been divided into two steps. In step 1, the P values in all rules (under the condition $N_{case,i}/N_{case} > N_{control,i}/N_{control}$) 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).

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

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.

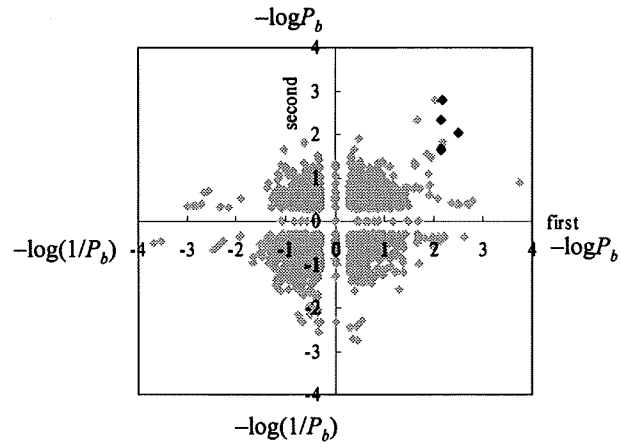

FIG. 3. Relationship of P value in same rule between first- and second-set samples in up to three single nucleotide polymorphism (SNP) combinations. Rules with a higher control subject rate than that of population data were plotted in the more than 0 area on the $-\log P_b$ axis, whereas those with a 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.