by novelty stress. Further in vivo study using GTP-CH1 inhibitors will help to clarify the mechanisms of these BH_4 stress responses. Although 5-HT turnover was enhanced by novelty stress in the midbrain of the mice exposed to the group-housing condition, novelty stress did not change 5-HT turnover in the same region of the brain in the mice exposed to the isolation-housing condition. Thus, social isolation suppressed the increase in 5-HT turnover elicited by acute environmental stress in the midbrain.

Although the results indicating the influence of social isolation on changes in BH_4 levels elicited by novelty stress were somewhat different from those of our recent studies (Miura et al., 2005a), the discrepancy may have been because of differences in animal age and the stress protocol of the two studies. In the present study, mice were isolated at 9 weeks of age and reared for 28 days, whereas they were isolated at 7 weeks and reared for 35 days in our recent study (Miura et al., 2005a). Neurobiological development may therefore have been differed in the two studies. The novelty stress session consisted of one 20-min exposure in the present study, whereas two 10-min habituation sessions, followed by one 20-min true stress session, were used in our recent study. Thus, the present study utilized a more "acute" and "novel" stress procedure. We assume that the influence of "novelty" stress was more precisely detectable in the present study because of this revision of the protocol.

As mentioned above, social isolation altered the response to acute environmental stress, as determined by changes in BH_4 levels and 5-HT turnover. The BH_4 response to stress was enhanced in both regions of the brain examined, i.e., in the prefrontal cortex and midbrain, whereas the increased 5-HT turnover response to stress was suppressed in the midbrain. These results suggest that social isolation strengthens the BH_4 biosynthetic response to novelty stress, suppressing 5-HT release and reuptake in response to the stress. In other words, social isolation modified neuronal activity in response to the stress. Furthermore, social isolation altered the effects of paroxetine on BH_4 levels, as well as DA and 5-HT turnover. Social isolation shifted the main effects of paroxetine, which were to attenuate DA and 5-HT turnover, to a suppression of BH_4 elevation, which had been elicited by novelty stress. The underlying mechanism responsible for this shift remains unknown. However, the effects of paroxetine changed in such a manner that the expected changes (i.e., elevated BH_4 levels elicited by social isolation and novelty stress) were suppressed. These findings suggest that two environmental factors, namely, social isolation and novelty stress, which are known to be closely related to the etiology and pathophysiology of major depression, elicited modifications in neurochemical activity and shifted the effects of paroxetine, thus countering the neurochemical changes elicited by these factors. Investigation of the underlying mechanisms responsible for the modification of BH_4 levels by environmental factors will help clarify the neuropharmacological regulation of major depression by SSRIs.

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Article

Identification of Functional Polymorphisms in the Promoter Region of the Human PICK1 Gene and Their Association With Methamphetamine Psychosis

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Objective: Protein interacting with C-kinase-1 (PICK1) plays a role in the targeting and clustering of dopamine transporter, which is the primary target site for the abused drug methamphetamine. Based on the interaction of PICK1 with dopamine transporter, it is of particular interest to investigate the association between the PICK1 gene and methamphetamine abusers.

Method: The authors studied the association between PICK1 gene polymorphisms and methamphetamine abusers in a Japanese group. Two hundred and eight methamphetamine abusers and 218 healthy comparison subjects were enrolled in the study. Furthermore, the authors also examined the effects of single nucleotide polymorphisms (SNPs) in the promoter and 5′-untranslated region on transcription levels of PICK1.

Results: The authors identified four highly frequent SNPs, rs737622 (–332 C/G) and rs3026682 (–205 G/A) in the promoter region and rs713729 (T/A) in intron3 and rs2076369 (T/G) in intron4. Of these SNPs, rs713729 was significantly associated with methamphetamine abusers in general, and rs713729 and rs2076369 were significantly associated with those with spontaneous relapse of psychosis. Furthermore, haplotype analysis revealed that specific haplotypes of these SNPs were associated with methamphetamine abusers. A gene reporter assay revealed that the two SNPs in the promoter region significantly altered transcriptional activity.

Conclusions: Our findings suggest that the PICK1 gene may be implicated in the susceptibility to spontaneous relapse of methamphetamine psychosis and that, as an intracellular adapter protein, PICK1 may play a role in the pathophysiology of methamphetamine psychosis.

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Methamphetamine is one of the most widely used illicit drugs, and its abuse continues to be a growing problem worldwide. Accumulating evidence has suggested that genetic factors play a role in vulnerability to methamphetamine abuse and the psychiatric symptoms related to methamphetamine abuse (1–5). The principal target for the action of methamphetamine is the dopamine transporter, which removes dopamine from the extracellular space at the synapse and thereby controls dopamine signals (6, 7). Both the activity and the surface availability of the dopamine transporter are believed to be tightly regulated by different cellular mechanisms, the best characterized being modulation by protein kinase C activation (8, 9). Recent positron emission tomography (PET) studies of methamphetamine abusers have demonstrated that the density of dopamine transporter is significantly low in the caudate/putamen of methamphetamine abusers (10, 11), suggesting that the long-term use of methamphetamine leads to damage of dopaminergic neurons in the human brain. Of interest, the variable number of tandem repeats polymorphism of the human dopamine transporter gene has been shown to be a risk factor for a prognosis of prolonged-type methamphetamine psychosis (12).

A protein interacting with C kinase (PICK1), one of the PSD95/disk-large/ZO-1 (PDZ) domain-containing synaptic proteins, was originally identified by a yeast two-hybrid system on the basis of its interaction with protein ki-

This article is featured in this month's AJP Audio and is discussed in an editorial by Dr. McMahon on p. 999.

TABLE 1. Demographic and Clinical Characteristics of Comparison Subjects and Methamphetamine Abusers

Variable	Comparison Subjects			Methamphetamine Abusers			p
	N			N			
Sex (men/women)	175/43			169/39			0.81[a]
Prognosis of psychosis				178			
Transition type				100			
Prolonged type				78			
Spontaneous relapse							
Positive				77			
Negative				118			
Polysubstance abuse							
No				55			
Yes				140			
	Mean	SD	Range	Mean	SD	Range	p
Age (years)	39.0	12.3	19–73	36.9	11.3	18–69	0.29[b]

[a] Chi-square test.
[b] t test.

nase C alpha (13, 14). PICK1 plays a role in the targeting and, when serving as a scaffold, in the localization of synaptic membrane proteins such as the dopamine transporter (15). PICK1 interacts with dopamine transporter through the PDZ domain of PICK1 and the last three residues of the carboxyl terminal of dopamine transporter (16). Thus, it is likely that the interaction of PICK1 with dopamine transporter results in a clustering of dopamine transporter on the cell surface and a subsequent enhancement of dopamine transporter uptake activity due to an increase in plasma membrane dopamine transporter density in mammalian cells and dopamine neurons in culture.

The PICK1 gene has been mapped to chromosome 22q13.1, a region thought to contain a gene for schizophrenia (17). It is well known that methamphetamine psychosis is similar to the psychosis associated with schizophrenia (18). In a case-control study, Hong et al. (19) reported that the PICK1 gene was associated with schizophrenia in the Taiwanese population. Furthermore, in a case-control association study with well-characterized Japanese subjects, Fujii et al. (20) reported an association of the PICK1 gene with schizophrenia, which is more prominent in people with the disorganized type of schizophrenia. Taken together, these findings point to the possibility of an association between the PICK1 gene and methamphetamine psychosis.

The present study was undertaken to examine the association between PICK1 gene polymorphisms and methamphetamine abuse. Using a gene reporter assay, we also investigated the effects of the single nucleotide polymorphisms (SNPs) in the promoter and 5′-untranslated regions on the levels of PICK1 transcription.

Materials and Methods

Subjects

The subjects were 208 patients (169 men and 39 women, ages: mean=36.9 years, SD=11.3, age range=18–69) with methamphetamine dependence and a psychotic disorder meeting the ICD-10-DCR criteria (F15.2 and F15.5) who were outpatients or inpatients of psychiatric hospitals affiliated with the Japanese Genetics Initiative for Drug Abuse and 218 age-, gender-, and geographical origin-matched normal comparison subjects (175 men and 43 women, age: mean=39.0 years, SD=12.3, age range=19–73) with no past history and no family history of drug dependence or psychotic disorders (Table 1). The age of the normal subjects did not differ from that of the methamphetamine abusers (Table 1). The research was performed after approval was obtained from the ethics committees of each institute of the Japanese Genetics Initiative for Drug Abuse, and all subjects provided written informed consent for the use of their DNA samples as part of this study.

Background of Methamphetamine Abusers

Diagnoses were made by two trained psychiatrists based on interviews and available information, including hospital records. Subjects were excluded if they had a clinical diagnosis of schizophrenia, another psychotic disorder, or an organic mental syndrome. All subjects were Japanese and were born and living in restricted areas of Japan, including northern Kyushu, Setouchi, Chukyo, Tokai, and Kanto. The patients were divided into subgroups by characteristic clinical features (Table 1).

Prognosis of Psychosis

The prognosis of methamphetamine psychosis varied among patients, some of whom showed continued psychotic symptoms, even after methamphetamine discontinuance, as previously reported (21, 22). Accordingly, the patients were categorized by prognosis into two groups, a transient type and a prolonged type, based on the duration of the psychotic state after methamphetamine discontinuance. The transient type is defined as those whose symptoms improved within 1 month, and the prolonged type is those whose psychosis continued for more than 1 month after methamphetamine discontinuance and the start of treatment *with neuroleptics*. In this study, there were 100 transient type and 78 prolonged type patients with methamphetamine psychosis (Table 1). One of the issues in categorizing was the difficulty in distinguishing patients who coincidently developed schizophrenia. Therefore, we excluded cases in which the predominant symptoms were of the negative and/or disorganized type in order to maintain the homogeneity of the subgroup.

Spontaneous Relapse

It has been well documented that once methamphetamine psychosis has developed, patients in a state of remission are susceptible to spontaneous relapse without reconsumption of methamphetamine (21, 22). It has thus been postulated that a sensitization phenomenon induced by the repeated consumption of methamphetamine develops in the brain of patients

TABLE 2. Polymerase Chain Reaction Primers Used to Search for Single Nucleotide Polymorphisms (SNPs) in 5′ Upstream Region and Exons of the PICK1 Gene and for Genotyping of SNP1-6

Region	Primer Sequences Forward (5′-3′)	Reverse (5′-3′)	Product size (bp)
5′-upstream-1	CACAATGTGGCTGGCAAGA	CCCCCCCTCCTTCCTTAGT	498
5′-upstream-2	CTCTGGGGAGCACTGATAGC	AGACACATGCCCTTTCACC	478
5′-upstream-3	GGGCCATTCTAGTAGGGGAGT	CAATCCCTGCAGACAATCCT	368
5′-upstream-4	GGGAAGGGAAGGATTATTGTCTGC	CAAGTGCCTAAATGCCAACGCC	395
Exon 2	GAGGGGTGGCGTTGGCATTTA	CACTGCTCCATCTGCTTTGCT	441
Exon 3	CAGTGGAGCCCCTCAGGAGTTTTAG	CAGGTGGTCAGAAAGCCCCTCTG	341
Exon 4	GAGCAGAGGGTAGAGTGGAAGAGG	ACAAGGAAGGGGGCGGTGAG	358
Exon 5	AGGAGTCTCAGTCCAGAACAGTCTTG	TTGGTCAGAGGTCAGAGCCCAC	301
Exon 6	CTCCCTGTGCATGGAGGTAAGG	TGGTGACTTCTCAGTTCCACGG	317
Exon 7	TGACCTCCCCTCTTCTTTGA	ATTTTGTAGGCTGGCATTCC	189
Exon 8	GGTTGGGTCGGACTGAGCTTTTAC	AGCTTTGGGGGATGCCATTACC	256
Exon 9	GCTTCTCCCCAACAAACCCCTG	CTCCAGCATACGACCTTCTCTGC	295
Exon 10	AGTCCACCAACAAGGGTGACGC	AGCATGGCTGACTGAAGTGGGG	263
Exon 11	GCCAGCCTCTCCTGCTGCGT	CCAGGAACGAGAGTCCAGCC	204
Exon 12	AGGTCTCAGGAATGAAGAACAGCC	TTTCCCACCTCTGAAATGGAGAG	288
Exon 13-1	GAGAGTCTCCTCCCTGAGGC	CTCCTTCCTAAGGCAGGTCC	729
Exon 13-2	AGAGGGAGAGCTTGGTCTCTGGACC	AAGGAGGGTCTGAAGCCACTGCGAC	358
SNP[a]	Primer or probe sequences forward primer (5′-3′) or probe 1 (5′-3′)	Reverse primer (5′-3′) or probe 2 (5′-3′)	Product size (bp)
SNP1 (rs737622)	TCCGGACTCAATTAGCCACCTA; probe 1: VIC-CATATC-CCACGGCCGGT-MGB	GCCATGGAAGAAAGATACAGAAGGA; probe 2: FAM-CATATCCCACCGCCGGT-MGB	98
SNP2 (rs3026682)	CTGCCGGATGAGGTGGAT; probe 1: VIC-CTGGCTGTG-GCTCT-MGB	GCTGCCACTGCTATTGTGTAAAG; probe 2: FAM-CCTGGCTATGGCTCT-MGB	86
SNP3 (rs11089858)	GGCTCAGGGATGCTTTCGTT; probe 1: VIC-CGCGGGC-CCCTGA-MGB	GGGTTTGTCCCAGCTTCCT; probe 2: FAM-CGCG-GACCCCTGA-MGB	83
SNP4 (rs713729)	CCAGTACT GTCCCTGCCTCT	TAAGTGCCGAGAAGGAAAAA	235
SNP5 (rs3952)	GGTCTTGCTTCTGCTCACAGT; probe 1: VIC-CCTCCT-TCATGAGCC-MGB	GGTCACAGGAGGCCGAAT; probe 2: FAM-CCTCCT-TCGTGAGCC-MGB	58
SNP6 (rs2076369)	CCAAATTGTTGGGATTACAGGT	GCTCTGACCAGCTTACCAATGT	220

[a] TaqMan 5′-exonuclease allelic discrimination assay was used for the genotyping of SNP1-3 and 5, and direct sequencing was used for the genotyping of SNP4 and 6.

with methamphetamine psychosis, which provides a neural basis for an enhanced susceptibility to relapse. Therefore, the patients in this study were divided into two groups according to the presence or absence of spontaneous relapse. In this study, 77 patients underwent a spontaneous relapse, and 118 did not (Table 1).

Polysubstance Abuse

The patients were divided according to polysubstance abuse status; 55 patients had abused only the drug methamphetamine in their lifetime, and 140 patients had abused both methamphetamine and other drugs in the present or past. After methamphetamine abuse, organic solvents and marijuana were the most frequently used substances. Cocaine and heroin were rarely abused in this group of subjects.

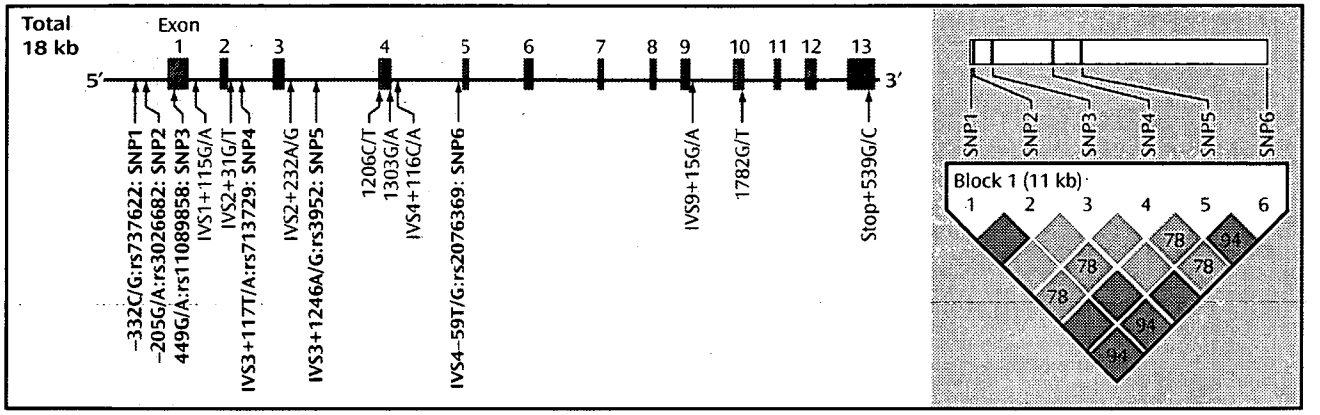
Identification of SNPs

The association between the SNPs of the PICK1 gene and schizophrenia has been reported by two groups. Hong et al. (19) reported a case-control study of the PICK1 gene polymorphism (rs3952) and schizophrenia patients in a Chinese sample. In a Japanese sample, Fujii et al. (20) demonstrated an association between two SNPs (rs713729 and rs2076369) of the PICK1 gene and schizophrenia. However, it remained unclear whether highly common SNPs exist in the 5′-upstream region and the exons of the PICK1 gene in the Japanese population. Therefore, we searched for SNPs in the 5′-upstream region and in all 13 exons with the flanking intronic region of the PICK1 gene using a direct sequencing method. We designed a total of 34 primers for polymerase chain reactions (Table 2) based on information about the PICK1 gene obtained from a public database (the PICK1 gene sequence was assigned as a portion of AL031587, May 18, 2005, i.e., as protein kinase C alpha binding protein; http://www.ncbi.nlm.nih.gov/). Amplification was carried out with an initial denaturation at 95°C for 1 minute, followed by 40 cycles at 95°C for 1 minute, 60°C for 1 minute, and 72°C for 40 seconds, with a final extension at 72°C for 5 minutes. The sequencing reaction was performed on an ABI 310 genetic analyzer (PE Biosystems, Foster City, Calif.) following the manufacturer's protocol.

For the screening of the 5′-upstream region, pairs of polymerase chain reaction primers were designed to amplify 368–498-bp fragments in approximately 1000 bp of the 5′-upstream region (Table 2). To determine the transcription start position, we used a large-insert cDNA library made from human fetal brain (Clontech Laboratories, Inc., Mountain View, Calif.). Based on SMART technology (Clontech), the cDNA library contains high-fidelity full-length transcripts. We performed polymerase chain reactions with 5′-sequencing primer supplied by the manufacturer and the 5′-3R primer we designed in our laboratory (Table 2). By using a TOPO TA cloning kit (Invitrogen, Carlsbad, Calif.), the polymerase chain reaction product was cloned into TA plasmids according to the manufacturer's instructions. Then the inserted 5′-upstream region was direct-sequenced with sequencing primers provided with the TA cloning kit.

For all polymerase chain reaction products, we first analyzed the sequences of the 32 comparison subjects, and we identified three SNPs in the 5′-upstream region and 11 SNPs in the exons and their flanking intronic regions (Figure 1). Of these 14 SNPs, minor allele frequencies of two SNPs in the 5′-upstream region and two SNPs in introns 3 and 4 were more than 10%. By referring to the dbSNP database (http://www.ncbi.nlm.nih.gov/SNP/), we confirmed that two of these SNPs in the 5′-upstream region were rs737622 (SNP1) and rs3026682 (SNP2) (Figure 1). Although none of the SNPs was described as highly frequent in all exons observed, we found that rs713729 (SNP4) in intron 3 and rs2076369 (SNP6) in intron 4 were highly frequent; these re-

FIGURE 1. Genomic Structure and Location of Polymorphic Sites of the PICK1 Gene[a]



[a] The rectangles and horizontal lines represent exons and introns, respectively. Of these single nucleotide polymorphisms (SNPs), six (SNPs 1–6, indicated in boldface) were highly frequent. The haplotype block structure with linkage disequilibrium parameters D′ is shown in the right hand panel. The D′ values were calculated from comparison groups.

sults are in good agreement with those of a previous study (20) (Figure 1).

Genotyping of Identified SNPs

To investigate the putative association between PICK1 gene polymorphisms and methamphetamine abuse, we selected the following SNPs for genotyping: rs737622 (C/G: SNP1), rs3026682 (G/A: SNP2), rs110898858 (G/A: SNP3), rs713729 (T/A: SNP4), and rs2076369 (T/G: SNP6). To compare the present results with those of previous reports (19, 20), we also selected rs3952 (A/G: SNP5) for genotyping. For four of these SNPs, i.e., SNP1, 2, 3, and 4, genotyping was performed by TaqMan 5′-exonuclease allelic discrimination assay in accordance with the manufacturer's protocol. The primers and probes used for these SNPs are shown in Table 2.

For SNP4 (rs713729) and SNP6 (rs2076369), genotyping was performed by direct sequencing, and the primers used for polymerase chain reactions are shown in Table 2.

Dual-Luciferase Gene Reporter Assays

Reporter plasmids containing the rs737622 (-332C/G: SNP1), rs3026682 (-205G/A: SNP2), and rs11089858 (449G/A: SNP3) polymorphic sites were constructed, and 1039-bp fragments (from −373 to +666, Figure 2) were amplified from the genomic DNAs with the identified genotypes as templates. The polymerase chain reaction primers were as follows: forward, 5′-CGACGCGTCCGGACTCAATTAGCCACCT-3′ (including a MluI site) and reverse, 5′-CGCTCGAGTCGGAACCAAGAACGAGAAC-3′ (including an XhoI site). The polymerase chain reaction products of four haplotypes (C-332/G-205/G+449: Pr1, C-332/G-205/A+449: Pr2, G-332/A-205/A+449: Pr3, and G-332/A-205/A+449: Pr4) were cloned into the pGL-3 Basic Plasmid (Promega Corporation, Madison, Wis.). The inserted sequences were confirmed with direct sequencing by using an ABI 310 genetic analyzer (PE Biosystems, Foster City, Calif.) according to the manufacturer's protocol.

Two cell lines, human neuroblastoma SK-N-SH and human glioblastoma U-87, were cultured in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum. Luciferase reporter plasmids containing the four haplotypes were transiently transfected into these cells by using the TransFast lipofection reagent (Promega Corporation, Madison, Wis.). The renilla luciferase expression plasmid phRL-TK was cotransfected as an internal standard. After 48 hours, the cells were harvested, and the luciferase reporter activity was measured by using a TD-20/20 luminometer and a Dual-Luciferase Assay Kit (Promega Corporation, Madison, Wis.). All experiments were repeated at least three times.

Statistical Analysis

Allele and genotype frequencies were calculated, and the differences between groups were evaluated with Fisher's exact test. Case-control haplotype analysis was performed by the maximum-likelihood method by using SNPAlyse (DYNACOM, Yokohama, Japan, http://www.dynacom.co.jp/); p values of haplotypes were obtained by 1000-fold permutation to correct for bias due to multiple tests. For the luciferase assay, one-way analysis of variance (ANOVA) followed by post hoc Bonferroni tests were performed for comparison of relative luciferase activity among four types of inserted vectors. The analysis was performed with SPSS software (SPSS version 12.0J, Tokyo). All statistically significant p values were set at <0.05.

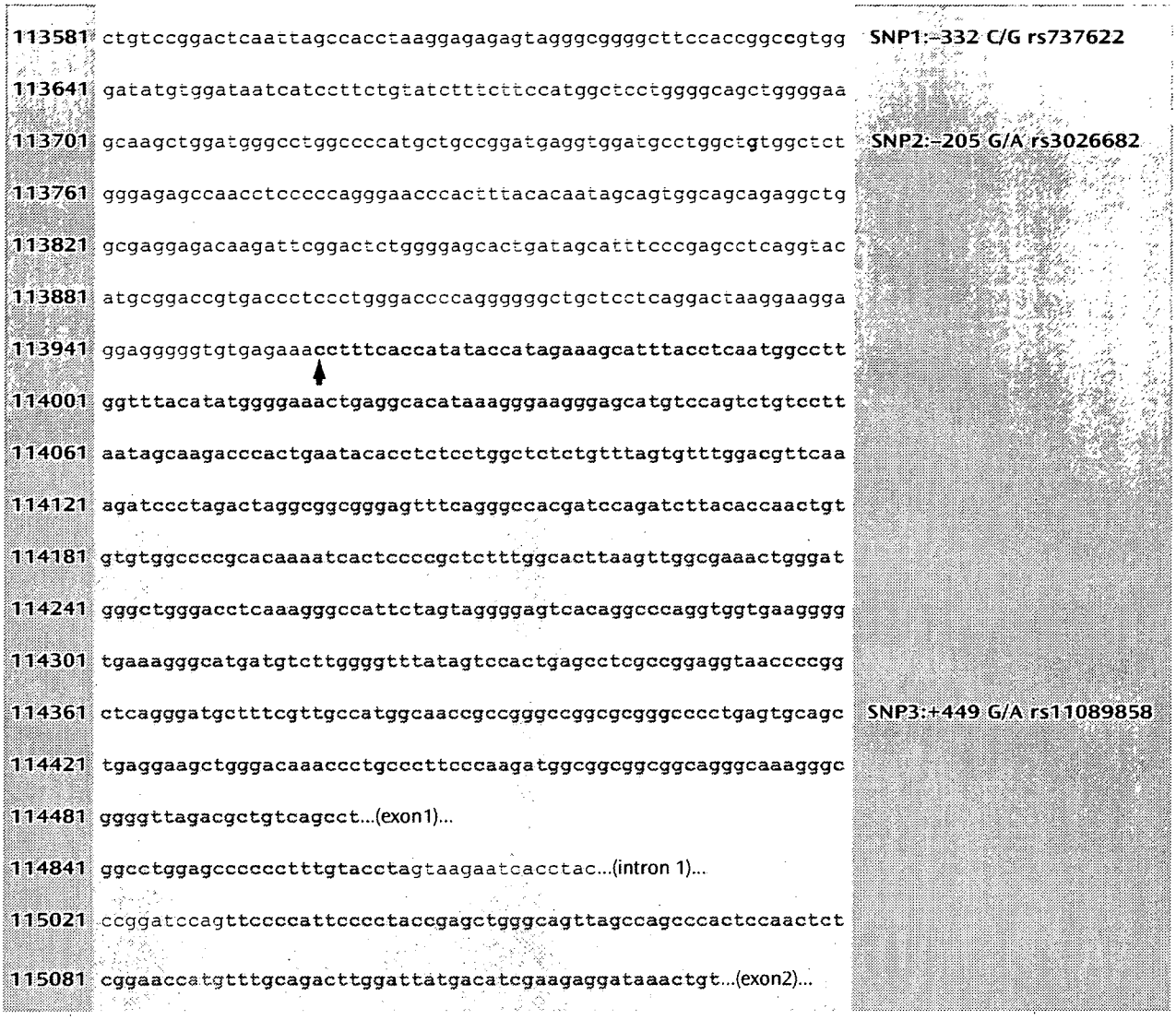
Results

Identification of SNPs and Association Studies

In searching the transcription start position, we found that exon 1 turned out to stretch beyond the position reported in the public database (Figure 2). Namely, we found that the transcription start position was at 113958, which is 513 bp before the start position (114471) reported in AL031587 (http://www.ncbi.nlm.nih.gov/).

We searched for the SNPs in the PICK1 gene, including the promoter region approximately 500 bp ahead of the transcription start position, the entire 5′-untranslated sequence from the translation start position in exon 2, and all 13 exons and their neighboring sequences. In this study, we found 14 SNPs in the PICK1 gene (Figure 1). Of these SNPs, rs737662 (-332C/G: SNP1), rs3026682 (-205G/A: SNP2), rs11089858 (449 G/A: SNP3), rs713729 (IVS3+117T/A: SNP4), and rs2076369 (IVS4-59T/G: SNP6) were found to be highly frequent (the minor allele >10%) (Figure 1). Subsequent genotyping was performed for these five SNPs (SNP1, 2, 3, 4, and 6) and rs3952 (IVS3+1246A/G: SNP5). Both the genotype and the allele

FIGURE 2. Schematic Diagram of 5′-Upstream Region of the PICK1 Gene[a]



[a] The numbers indicate the nucleotide positions cited from the NCBI database AL031587. A bold black arrow indicates the transcription start position we identified, which was 513 bp before the start position (114471) reported in the database. Blue characters indicate exons of PICK1, and the translation start codon, ATG, is orange. The positions of the three SNPs we identified are indicated in red.

distributions of SNP1, SNP2, and SNP5 were completely the same (Table 3). The allele frequencies and genotype distributions of SNP1, 3, 4, and 6 in methamphetamine abusers and comparison subjects are shown in Table 3. The genotype distributions were within the Hardy-Weinberg equilibrium.

We found significantly different frequencies between comparison subjects and methamphetamine abusers in SNP4 (Table 3). The frequency (88.7%) of carrying the T allele among the methamphetamine abusers was significantly higher (odds ratio=1.58, 95% confidence interval [CI]=1.06–2.34, $p<0.03$) than that of the comparison subjects (83.3%), and we also detected a different distribution of genotype ($p<0.03$). Positive associations were detected in the subgroup of those who experienced psychosis (alleles, $p=0.007$, odds ratio=1.79, 95% CI=1.17–2.74, genotype, $p<0.02$), transient-type psychosis (alleles, $p=0.01$, odds ratio=2.03, 95% CI=1.17–3.51, genotype, $p<0.03$), and psychosis with spontaneous relapse (alleles, $p=0.003$, odds ratio=2.61, 95% CI=1.35–5.07, genotype, $p=0.004$) and in abusers without polysubstance abuse (alleles, $p<0.03$, odds ratio=2.26, 95% CI=1.09–4.67, genotype, $p<0.04$) (Table 3). For SNP6, the frequency (48.7%) of the T allele among methamphetamine abusers who experienced psychosis with spontaneous relapse was significantly higher (odds ratio=1.62, 95% CI=1.19–2.35, $p<0.02$) than that of the comparison subjects (36.9%), and we also detected a different distribution of genotype ($p<0.02$) (Table 3). In contrast, no differences for SNP1, 2, 3, and 5 were detected between methamphetamine abusers and comparison subjects (Table 3).

TABLE 3. Genotypic and Allelic Distributions of the PICK1 Gene Polymorphisms in Comparison Subjects and Methamphetamine Abusers

Variable		Genotype							Allele				
		C/C		C/G		G/G			C		G		
SNP1[a] (rs737622)	N	N	%	N	%	N	%	p[b]	N	%	N	%	p[b]
Comparison subjects	218	89	40.8	107	49.1	22	10.1		285	65.4	151	34.6	
Methamphetamine abusers	208	85	40.9	93	44.7	30	14.4	0.35	263	63.2	153	36.8	0.52
Psychosis	178	66	37.1	87	48.9	25	14.0	0.45	219	61.5	137	38.5	0.27
Transient	100	38	38.0	48	48.0	14	14.0	0.56	124	62.0	76	38.0	0.42
Prolonged	78	28	35.9	39	50.0	11	14.1	0.53	95	60.9	61	39.1	0.33
Spontaneous relapse													
Positive	77	32	41.6	33	42.9	12	15.6	0.37	97	63.0	57	37.0	0.62
Negative	118	48	40.7	55	46.6	15	12.7	0.73	151	64.0	85	36.0	0.74
Polysubstance abuse													
No	55	23	41.8	23	41.8	9	16.4	0.35	69	62.7	41	37.3	0.66
Yes	140	58	41.4	63	45.0	19	13.6	0.53	179	63.9	101	36.1	0.75
		G/G		G/A		A/A			G		A		
SNP3 (rs11089858)	N	N	%	N	%	N	%	p[b]	N	%	N	%	p[b]
Comparison subjects	218	180	82.5	37	17.0	1	0.5		397	91.1	39	8.9	
Methamphetamine abusers	208	167	80.3	39	18.8	2	1.0	0.71	373	89.7	43	10.3	0.56
Psychosis	178	143	80.3	34	19.1	1	0.6	0.80	320	89.9	36	10.1	0.63
Transient	100	81	81.0	19	19.0	0	0.0	0.83	181	90.5	19	9.5	0.88
Prolonged	78	62	79.5	15	19.2	1	1.3	0.47	139	89.1	17	10.9	0.52
Spontaneous relapse													
Positive	77	64	83.1	13	16.9	0	0.0	1.00	141	91.6	13	8.4	1.00
Negative	118	94	79.7	23	19.5	1	0.8	0.65	211	89.4	25	10.5	0.49
Polysubstance abuse													
No	55	44	80.0	11	20.0	0	0.0	0.75	99	90.0	11	10.0	0.71
Yes	140	112	80.0	26	18.6	2	1.4	0.58	250	89.3	30	10.7	0.44
		T/T		T/A		A/A			T		A		
SNP4 (rs713729)	N	N	%	N	%	N	%	p[b]	N	%	N	%	p[b]
Comparison subjects	218	150	68.8	63	28.9	5	2.3		363	83.3	73	16.7	
Methamphetamine abusers	208	166	79.8	37	17.8	5	2.4	<0.03	369	88.7	47	11.3	<0.03
Psychosis	178	145	81.5	30	16.9	3	1.7	<0.02	320	89.9	36	10.1	0.007
Transient	100	83	83.0	16	16.0	1	1.0	<0.03	182	91.0	18	9.0	0.01
Prolonged	78	62	79.5	14	17.9	2	2.5	0.14	138	88.5	18	11.5	0.15
Spontaneous relapse													
Positive	77	67	87.0	9	11.7	1	1.3	0.004	143	92.9	11	7.1	0.003
Negative	118	88	74.6	26	22.0	4	3.4	0.36	202	85.6	34	14.4	0.51
Polysubstance abuse													
No	55	47	85.5	7	12.7	1	1.8	<0.04	101	91.8	9	8.2	<0.03
Yes	140	109	77.9	28	20.0	3	2.1	0.16	246	87.9	34	12.1	0.11
		G/G		G/T		T/T			G		T		
SNP6 (rs2076369)	N	N	%	N	%	N	%	p[b]	N	%	N	%	p[b]
Comparison subjects	218	82	37.6	111	50.9	25	11.5		275	63.1	161	36.9	
Methamphetamine abusers	208	73	35.1	99	47.6	36	17.3	0.23	245	58.9	171	41.1	0.23
Psychosis	178	64	36.0	83	46.6	31	17.4	0.25	211	59.3	145	40.7	0.30
Transient	100	34	34.0	48	48.0	18	18.0	0.30	116	58.0	84	42.0	0.25
Prolonged	78	30	38.5	35	44.9	13	16.7	0.41	95	60.9	61	39.1	0.63
Spontaneous relapse													
Positive	77	21	27.3	37	48.1	19	24.7	<0.02	79	51.3	75	48.7	<0.02
Negative	118	46	37.9	56	47.5	16	13.6	0.77	148	62.7	88	37.3	0.93
Polysubstance abuse													
No	55	15	27.3	30	54.5	10	18.2	0.23	60	54.5	50	45.5	0.13
Yes	140	53	37.9	62	44.3	25	17.9	0.19	168	60.0	112	40.0	0.43

[a] The distributions of SNP2 (rs3026682) and 5 (rs3952) are the same as SNP1 (rs737622).
[b] Versus comparison subjects.

TABLE 4. Haplotype Analysis of Six Single Nucleotide Polymorphisms

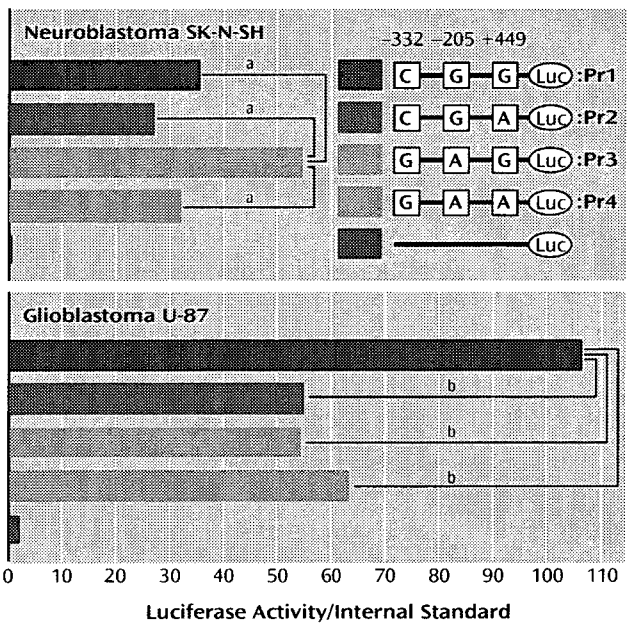
Variable	Haplotype Analysis		
Overall			
Haplotype	Comparison Subjects (N=218)	Methamphetamine Abusers (N=208)	p
C-G-G-T-A-T	35.2%	33.7%	0.63
G-A-G-T-G-G	32.3%	32.3%	0.85
C-G-G-A-A-G	14.5%	9.2%	<0.02
C-G-A-T-A-G	8.3%	7.4%	0.66
C-G-G-T-A-G	5.5%	8.9%	<0.09
G-A-G-T-G-T	0.7%	3.5%	0.01
C-G-G-A-A-T	1.2%	1.7%	0.66
G-A-G-A-G-G	1.0%	0.4%	0.40
Methamphetamine abusers			
Haplotype	With Spontaneous Relapse (N=77)	Without Spontaneous Relapse (N=117)	p
C-G-G-T-A-T	42.3%	27.8%	0.001
G-A-G-T-G-G	32.1%	31.1%	0.86
C-G-G-A-A-G	4.5%	12.6%	<0.02
C-G-A-T-A-G	6.8%	6.3%	0.82
C-G-G-T-A-G	6.3%	11.8%	0.14
G-A-G-T-G-T	2.5%	4.9%	0.31
C-G-G-A-A-T	2.5%	1.3%	0.54

As shown in Figure 1, a strong linkage disequilibrium was observed in five of these six SNPs. Two haplotypes, C(SNP1)-G(SNP2)-G(SNP3)-A(SNP4)-A(SNP5)-G(SNP6) and G(SNP1)-A(SNP2)-G(SNP3)-T(SNP4)-G(SNP5)-T(SNP6), were significantly different between comparison subjects and methamphetamine abusers (Table 4). The frequency (9.2%) of the CGGAAG haplotype in the methamphetamine abusers was significantly lower (odds ratio= 0.60, 95% CI=0.45–0.79, p<0.02) than that of the comparison subjects (14.5%), and the frequency (3.5%) of the GAGTGT haplotype in the methamphetamine abusers was significantly higher (odds ratio=5.2, 95% CI=2.27–11.6, p=0.01) than that (0.7%) of the comparison subjects (Table 4). Of interest, a haplotype analysis between methamphetamine abusers with and without spontaneous relapse of psychosis showed the significant difference in the most major haplotype (CGGTAT) as well as the CGGAAG type. The frequency (42.3%) of CGGTAT type in the methamphetamine abusers with spontaneous relapse was significantly higher (odds ratio=2.2, 95% CI=1.80–2.61, p= 0.001) than that in those without spontaneous relapse (27.8%) (Table 4). As to the frequency of the CGGAGG type, the frequency (4.5%) in methamphetamine abusers with spontaneous relapse was significantly lower (odds ratio= 0.33, 95% CI=0.23–0.47, p<0.02) than that in those without spontaneous relapse (Table 4).

Transcriptional Effects of SNPs in the Promoter Region

The transcriptional effects of four promoter haplotypes on SK-N-SH cells and U-87 cells were also examined. As shown in Figure 3, the results for these two cell lines differed. For SK-N-SH cells, a substitution variant, Pr3 (G-332/A-205/A+449), showed significantly increased relative luciferase activity (1.54 for Pr3/Pr1, p<0.001, 2.03 for Pr3/Pr2, p<0.001, 1.74 for Pr3/Pr4, p<0.001). In contrast, for U-87 cells, every substitution showed significantly lower relative luciferase activity than that of the major type, Pr1 (C-332/G-205/G+449) (0.51 for Pr2/Pr1, p<0.001, 0.51 for Pr3/Pr1, p<0.001, 0.59 for Pr4/Pr1, p<0.001).

FIGURE 3. Relative Luciferase Activity of the Four Haplotypes in SK-N-SH Cells (top) and U-87 Cells (bottom)[a]



[a] The phRL-TK vector used was a negative control. The pGL3 Basic vector, which does not contain any promoter sequences, was used as a negative control. Each value is shown as the mean for three independent experiments.
[b] p<0.001.

Discussion

The major findings of the present study were the discovery of an association between PICK1 gene polymorphisms and methamphetamine abusers and the identification of functional SNPs (SNP1 and SNP2) in the promoter region of the PICK1 gene. It was of great interest to find that SNP4 and SNP6 were significantly associated with methamphet-

amine abusers who experienced spontaneous relapse of psychosis. In addition, the haplotype analysis demonstrated that specific haplotypes, C(SNP1)G(SNP2)G(SNP3)A(SNP4)A(SNP5)G(SNP6) and GAGTGT, were significantly associated with methamphetamine abusers in general. Furthermore, we also found that the frequencies of major haplotypes CGGTAT and CGGAAG were significantly different between methamphetamine abusers with and without spontaneous relapse of psychosis. Spontaneous relapse of psychosis among methamphetamine abusers is known as "flashbacks," which are known to follow nonspecific stress, even after the consumption of methamphetamine has ceased and drug treatment has begun, and it appears that a psychotic state might be induced by excess dopaminergic activity (21, 22). Given the role of dopamine systems in the pathogenesis of methamphetamine psychosis, it is possible that a functional alteration of dopamine transporter may be caused by genetic variations in PICK1 and can lead to dysfunction of the dopamine system. Taken together, these results suggest that the CGGTAT and CGGAAG haplotypes in the PICK1 gene are likely to be associated with the psychosis of methamphetamine abusers who experience spontaneous relapse. The different distributions of those two haplotypes between methamphetamine abusers with and without spontaneous relapse of psychosis also suggest the difference in genetic backgrounds between the two groups. In the present study, the group of subgroups was small. Because of the small size of subcategories, type I error cannot be ruled out. Therefore, further studies with a large group with subcategories would reveal the associations between the PICK1 gene and methamphetamine-induced psychosis.

In the 5′-upstream region of the PICK1 gene, we identified three SNPs (SNP1: −332 C/G, rs737622, SNP2: -205 G/A, rs3026682, and SNP3: 449G/A, rs11089858). A luciferase assay revealed the functional effects of these SNPs on transcriptional activities. Although the threshold scores were low, the TFSEARCH program (http://mbs.cbrc.jp/research/db/TFSEARCH.html) predicted that the major transcription factors, including GATA1 (for SNP1, score 78.3) and AML-1a (for SNP2, score 83.7), bind to either position of SNPs in the PICK1 promoter position. Of course, it is likely that unidentified transcription factors may also be involved in the transcriptional process because we found that the levels of PICK1 expression could be altered by nucleotide substitutions of these SNPs in the promoter region. After consideration of the role of PICK1 in the proper targeting and surface clustering of dopamine transporter (16), it is possible that altered PICK1 expression might lead to altered dopamine transporter function in synaptic dopamine signal transmission, which would in turn influence the pathogenesis of methamphetamine abuse and related psychotic symptoms.

In this study, we found that transcriptional effects of SNPs in the promoter region of the PICK1 gene differed in SK-N-SH and U-87 cells. The nucleotide substitutions (C→G at −332 and G→A at −205) showed significantly increased luciferase activity in SK-N-SH cells (neuronal cells), whereas the substitutions (C→G at −332 and G→A at −205) showed significantly decreased luciferase activity in U-87 cells (glial cells). Although the mechanisms underlying the discrepancy in these two cell lines are currently unknown, these findings suggest that PICK1 expression could be affected in different ways by these SNPs in neuronal and glial cells. Fujii et al. (20) reported that a haplotype, T(rs713729)-A(rs3952)-T(rs2076369), revealed a statistically significant association with disorganized schizophrenia in methamphetamine abusers in relation to comparison subjects ($p<0.02$). The TAT haplotype, discussed by Fujii and coworkers, was found to correspond to C(rs737622: SNP1)-G(rs3026682: SNP2)-G(rs11089858: SNP3)-T(rs713729: SNP4)-A(rs3952: SNP5)-T(rs2076329: SNP6) in our study, and it was the most frequent haplotype in both comparison subjects and methamphetamine abusers. As discussed, the frequency (42.3%) of the CGGTAT haplotype in methamphetamine abusers with spontaneous relapse was significantly higher ($p=0.001$) than that of those without spontaneous relapse (27.8%). These findings also suggest that methamphetamine abusers who experience a spontaneous relapse of methamphetamine psychosis might share a similar genetic susceptibility to schizophrenia.

It has been demonstrated that PICK1 interacts with other proteins, including AMPA receptors (14, 23) and metabotropic glutamate receptor 7 (mGluR7) (24, 25), which have been implicated in the pathophysiology of drug abuse as well as in schizophrenia (26–29). Thus, it seems that interactions of PICK1 with AMPA receptors and metabotropic glutamate receptors are likely to be involved in the pathogenesis of methamphetamine psychosis. Furthermore, Fujii et al. (20) identified PICK1 as a protein interactor with the D-serine synthesizing enzyme serine racemase in glial cells (30). After consideration of the role of D-serine in the pathophysiology of schizophrenia (31–35), it is likely that the interaction of PICK1 with serine racemase in glial cells may play a role in the pathophysiology of methamphetamine psychosis, although further studies will still be necessary.

In conclusion, the present findings revealed that PICK1 gene polymorphisms are associated with methamphetamine abusers, suggesting that the PICK1 gene plays a major role in a genetic susceptibility to methamphetamine psychosis.

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Association of *SOX*10 with schizophrenia in the Japanese population

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Background **Microarray studies of schizophrenic brains revealed decreases in the expression of myelin and oligodendrocyte-related genes. Of these genes, sex-determining region Y-box 10 (*SOX*10) is a major transcription factor modulating the expression of proteins involved in neurogenesis and myelination. The *SOX*10 gene is located on chromosome 22q13.1, a region repeatedly reported to show positive signals in linkage studies on schizophrenia.**

Objective **This study was conducted to clarify the exact role of *SOX*10 in the pathophysiology of schizophrenia.**

Methods **We performed an association analysis of *SOX*10 in a Japanese population of 915 schizophrenic patients and 927 controls. Genotyping was carried out using polymerase chain reaction restriction fragment length polymorphism.**

Main results **One single nucleotide polymorphism of the *SOX*10 gene (rs139887) was selected as a haplotype tag single nucleotide polymorphism using 96 controls. A significant association was observed in the genotype and allelic frequency of this single nucleotide polymorphism between schizophrenic patients and controls ($P = 0.025$ and $P = 0.009$, respectively). Especially, a significant association was found in male patients, but not female patients. We also performed a mutational search of the whole coding region, branch site, and promoter region of *SOX*10 in 96 schizophrenic patients, but no potential functional polymorphisms were detected.**

Conclusion **This study suggests that the *SOX*10 gene is related to the development of schizophrenia in the Japanese population. *Psychiatr Genet* 17:227–231 © 2007 Lippincott Williams & Wilkins.**

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Keywords: association study, myelin, oligodendrocyte, schizophrenia, single nucleotide polymorphism, *SOX*10

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Introduction

Functional abnormalities of neuronal connectivity have been reported in patients with schizophrenia (Liddle, 1996; McClure *et al.*, 1998). Histological, anatomical, and neuroimaging studies have led to the hypothesis that dysfunctional myelination during nerve fiber formation may contribute to the pathophysiology of schizophrenia (Davis *et al.*, 2003). Diffusion tensor imaging, which is considered to be a useful technique to estimate myelination *in vivo* (Klingberg *et al.*, 1999) showed decreased anisotropy in the prefrontal cortex, temporoparietal and parietooccipital regions, splenium, cingulum, and the posterior capsule and adjacent occipital white matter in schizophrenic patients compared with normal controls (Buchsbaum *et al.*, 1998; Lim *et al.*, 1999; Agartz *et al.*, 2001). In addition, several postmortem studies have demonstrated reductions in glial cell density (Rajkowska *et al.*, 1999) and neuronal size in the cerebral cortex of affected patients (Rajkowska *et al.*, 1999; Pierri *et al.*, 2001; Chana *et al.*, 2003). Thus, schizophrenic brain changes may occur not only in gray but also in white matter.

Recently, several groups identified decreased expression of myelin and oligodendrocyte-related genes in patients with schizophrenia or bipolar disorder using gene microarrays (Hakak *et al.*, 2001; Tkachev *et al.*, 2003; Dracheva *et al.*, 2006). Of these genes, the expression of sex-determining region Y-box 10 (*SOX*10), a major transcription-modulating factor involved in neurogenesis and myelination in the central and peripheral nervous systems (Kuhlbrodt *et al.*, 1998; Paratore *et al.*, 2001; Potterf *et al.*, 2001) was significantly decreased. In addition, dysfunction of *SOX*10 causes central dysmyelinating leukodystrophy

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(Pingault *et al.*, 1998), and up to 53% of patients with leukodystrophy concomitantly were reported to develop psychotic symptoms in adolescence and young adulthood (Hyde *et al.*, 1992).

The gene-encoding *SOX10* is located on chromosome 22q13.1; this locus includes a susceptibility gene locus linked to schizophrenia (Mowry *et al.*, 2004), which contains several candidate genes such as apolipoprotein L1 (*APOL1*) (Mimmack *et al.*, 2002), cytochrome P450 family 2 subfamily D polypeptide 6 (*CYP2D6*) (Fu *et al.*, 2006), phospholipase A2, group VI (*PLA2G6*) (Yu *et al.*, 2005), and protein interacting with PRKCA1 (*PICK1*) (Hong *et al.*, 2004).

Taken together, these data suggest that *SOX10* is a plausible candidate gene playing a role in the pathophysiology of schizophrenia, but the exact role of *SOX10* in the development of schizophrenia remains to be clarified. In this study, we performed a linkage disequilibrium (LD) analysis of *SOX10*, followed by case–control studies examining the association between *SOX10* polymorphism and schizophrenia using a large population.

Patients and methods

Patients

The participants consisted of 915 patients with schizophrenia (398 females: mean age ± standard deviation 50.1 ± 16.2; 517 males: 47.4 ± 14.8) and 927 controls (472 females: 38.5 ± 14.7; 455 males: 38.0 ± 14.3 years).

LD was evaluated using 96 control participants, whereas mutation screening was performed using 96 schizophrenic patients. Both of these subsets were randomly selected from the present participants. All participants were unrelated to each other and ethnically Japanese. A consensus diagnosis based on unstructured interviews was made for each patient by experienced psychiatrists according to the *Diagnostic and statistical manual of mental disorders*, 4th ed. (DSM-IV; American Psychiatric Association, 1994). All available medical records and information from family members were also taken into consideration. Mentally healthy controls had no current or past contact with psychiatric services based on unstructured interviews. This study was approved by the Ethics Committee of Nagoya University Graduate School of Medicine and Fujita Health University.

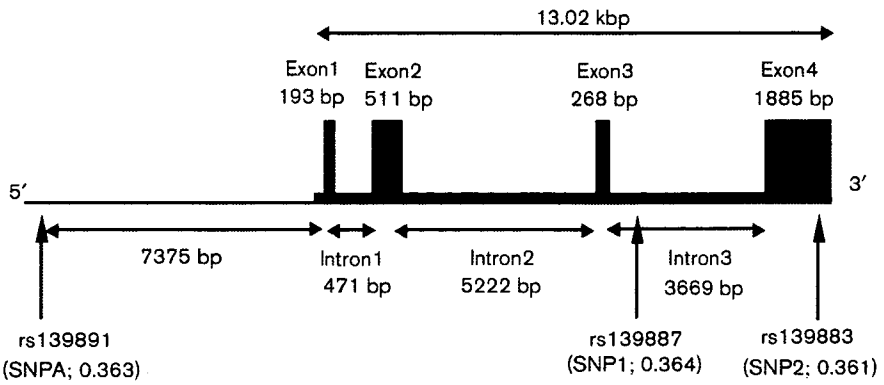
Genotyping

The *SOX10* gene is composed of four exons, spanning a total length of 13.02 kb. According to the information on *SOX10* genetic single nucleotide polymorphisms (SNPs) provided by the dbSNP database (*http://www.ncbi.nlm.nih.gov/SNP/*) we selected three SNPs, of which the minor allele frequencies are higher than 20%, for LD mapping analysis: SNPA (rs1398891 is 7375-bp upstream of *SOX10*), SNP1 (rs139887), and SNP2 (rs139883) (Fig. 1). Genomic DNA was extracted from the peripheral blood of all participants. Genotyping was performed by PCR restriction fragment length polymorphism (PCR-RFLP). Each primer and restriction enzyme used for the genotyping of SNPA, SNP1, and SNP2 by PCR-RFLP is described in Table 1.

Mutation search

Primer pairs were designed using information from GenBank sequence (accession number: NT_011520.10). Mutation search was performed in the whole coding regions, the promoter regions, the branch site, and 5′-flanking regions (500-bp upstream from the initial exons of the gene). Direct sequencing was performed using an ABI PRISM 3100 Genetic Analyzer (Applied Biosystems Tokyo, Japan Ltd).

Fig. 1



Genomic structure of *SOX10* with single nucleotide polymorphisms (SNPs) in linkage disequilibrium mapping. Numbers under the arrows represent SNP IDs. Parenthetic numbers represent the three SNPs that we selected and the minor allele frequencies (MAFs) of 96 controls.

Statistical analysis

Genotype deviation from the Hardy–Weinberg equilibrium (HWE) expectation was evaluated using the χ^2 test. The association between patients with schizophrenia and controls for genotype and allele distributions were also evaluated by χ^2 test. To evaluate pairwise LD matrices among SNPs (by D′ and r^2), we used HAPLOVIEW version 3.0 software (developed by Mark Daly; *http://www.broad.mit.edu/personal/jcbarret/haploview/index.php*). Haplotype tag SNPs (htSNPs) were then selected using the same program, as described by Johnson *et al.* (2001). The significance level for all statistical tests was set at 0.05.

Results

The genotype frequencies for all SNPs examined were in HWE, and all the D' values between the possible combinations of SNPs were 1.0. The r^2 values for SNPA–SNP1, SNPA–SNP2, and SNP1–SNP2 were 0.93, 0.96, and 0.95, respectively. As the *SOX10* genes are considered a single strong LD block, SNP1 was selected as an htSNP for this block.

The genotype distributions and allele frequencies of SNP1 in schizophrenic patients and controls are summarized in Table 2. The genotype frequency of SNP1 was not significantly different from the distribution expected from the HWE in 915 Japanese schizophrenic patients and 927 Japanese controls. The minor allele frequency decreased significantly in schizophrenic patients from that seen in normal controls. Association analysis revealed a significant difference in SNP1 genotype and allele distribution between controls and patients with schizophrenia ($P = 0.025$ and $P = 0.009$, respectively). Moreover, in view of the sex difference in gene effects, we included analyses of samples divided accordingly. As a result, significant association was found in SNP1 genotype and allele distribution between controls and male schizophrenic patients ($P = 0.010$ and $P = 0.006$, respectively), whereas no association was found in female patients.

No polymorphisms gave rise to nonsynonymous changes in the mutation search of 96 schizophrenic patients.

Discussion

In this study, we observed a significant difference in the minor allele frequency of SNP1 between schizophrenic patients and healthy controls. Our results suggest that the *SOX10* gene is related to the development of schizophrenia in the Japanese population, which may support the findings of microarray studies (Hakak *et al.*, 2001; Tkachev *et al.*, 2003) showing a significant decrease in the expression of the *SOX10* gene in the prefrontal cortex of postmortem brains in patients with schizophrenia.

Genetic variations in *SOX10* may influence the formation and maintenance of myelin sheath and may give rise to dysfunctional synaptic connectivity. These variations may be responsible for an individual patient's vulnerability for

Table 1 Primer and restriction enzymes used for genotyping

SNP	PCR primer sequence (5′–3′)	Product size (bp)	Annealing temperature	Restriction enzyme	Variants
rs139891 (SNPA)					
Forward	TTCCCATGCCTTCAGAGTTC	156	64	StyI	T/C
Reverse	TGCCTGCACACATTCTCTTC				
rs139887 (SNP1)					
Forward	GGGAGACCAGAGGAGGAGTC	180	64	HinfI	C/G
Reverse	CAGGGACACACACACACACA				
rs139883 (SNP2)					
Forward	TCCACTAAGTCCCTCGAACC	203	64	MspI	T/C
Reverse	GGAGGCCTTACCACTCCTATG				

Table 2 Genotypic analysis of SNP1 in SOX10 gene

		Genotype							
	Number	C/C	G/C	G/G	*P* value (genotype)	MAF	*P* value (allele)	OR	95% CI
Total									
Schizophrenia	915	456 (50%)	385 (42%)	74 (8%)	0.025	0.29	0.009	0.83	0.72–0.95
Control	927	405 (44%)	430 (46%)	92 (10%)		0.33			
Female									
Schizophrenia	398	192 (48%)	174 (44%)	32 (8%)	0.621	0.30	0.377	0.91	0.74–1.12
Control	472	217 (46%)	209 (44%)	46 (10%)		0.32			
Male									
Schizophrenia	517	264 (51%)	211 (41%)	42 (8%)	0.010	0.29	0.006	0.76	0.63–0.92
Control	455	188 (41%)	221 (49%)	46 (10%)		0.34			

CI, confidence interval; MAP, minor allele frequency; OR, odds ratio.

the development of schizophrenia. Oligodendrocytes function in the formation of myelin, express neurotransmitter receptors and facilitate interactions between neurons and glia during synaptic transmission (Haydon, 2001), and changes in *SOX*10 activity may cause alterations in neurotransmitter-triggered signal transduction and subsequent neuronal activity. Moreover, stratified by sex, only male patients showed significant association. This result could stem from different mechanism of myelination between sexes (Chambers and Perrone-Bizzozero, 2004). This speculation, however, needs further confirmation.

The SNP significantly associated with schizophrenia in this study is located within intron three of *SOX*10. As it is located outside the region affecting mRNA splicing, it seems unlikely to influence *SOX*10 function directly. An alternative possible interpretation is that the functional variant in LD with the minor allele of this SNP may work protectively against schizophrenia in controls. Considering that an actual susceptible polymorphism to schizophrenia may be located within the LD of this SNP, we examined a total of 96 patients with schizophrenia in this mutational search to detect a variant present at more than 5% with 95% power (Collins and Schwartz, 2002). No potentially functional polymorphism with a significant frequency was, however, found in this population.

The LD pattern observed in our sample was almost the same as that provided by the HapMap Project (HapMap data Release Number 16c.1: *http://www.hapmap.org/*). The block examined here covers the entirety of the *SOX*10 gene; however, the gene is included in a larger LD block according to the SNP browser data (SNP browser software version 3.0: *http://www.allsnps.com/snpbrowser*). Therefore, SNP1 may be in LD with an actual schizophrenia-susceptibility SNP located within another gene or noncoding DNA that can regulate gene transcription (Carninci *et al.*, 2005). To examine this possibility further, a complete mutational search of the genes surrounding *SOX*10 is needed. For example, polymerase (RNA) II (DNA-directed) polypeptide F (*POLR2F*), which is approximately 4 kbp downstream of *SOX*10, or protein interacting with PRKCA1 (*PICK1*), which is approximately 70 kbp upstream of *SOX*10, could be appropriate for these studies.

Several limitations could be considered in this study. First, there was a substantial demographic difference between the two groups and this may affect the present results. Second, the Japanese population is considered to be ethnically homogeneous; however, unidentified sample stratification could exist in this cohort, and we did not test for such stratification. Therefore, the undetectable results in our mutational search may reflect such latent sample stratification.

Recently, patients with schizophrenia were reported to exhibit increased *SOX*10 methylation, leading to reduced expression compared with control participants (Iwamoto *et al.*, 2005). Additionally, a striking contrast to our results, Iwamoto *et al.* (2006) reported that the genetic variations in the *SOX*10 gene do not contribute to susceptibility to Japanese schizophrenia. Further research is needed to elucidate the exact relationship between functional changes in the effects of *SOX*10 gene owing to the genetic variations and the pathophysiology of schizophrenia. Although our sample size is relatively large, replication of this study using independent sample sets will be required to confirm our findings.

Acknowledgements

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Association study between the *transferrin* gene and schizophrenia in the Japanese population

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Several lines of evidence, including diffusion tensor imaging and microarray studies, indicate that abnormalities in myelination play an important role in the pathophysiology of schizophrenia. Of myelin and oligodendrocyte-related genes, a significant decrease in the mRNA levels of transferrin in schizophrenics has been reported by both microarray and quantitative polymerase chain reaction studies. We performed an association analysis of the transferrin gene in a Japanese population of 384 schizophrenic patients and 384 controls. Six single nucleotide polymorphisms were genotyped by polymerase chain reaction-restriction fragment length polymorphism and a TaqMan assay. No significant differences in genotype, allele, or haplotype frequencies of the six single nucleotide polymorphisms were observed between schizophrenic patients and controls. The present results suggest that the *transferrin* gene is not related to the development of schizophrenia in the Japanese population. *NeuroReport* 18:517–520 © 2007 Lippincott Williams & Wilkins.

Keywords: association study, myelin, oligodendrocyte, schizophrenia, single nucleotide polymorphism, transferrin gene

Introduction

Numerous lines of evidence have suggested that disturbances in myelination contribute to the pathophysiology of schizophrenia. Several postmortem brain studies have demonstrated a reduction in glial cell density in affected patients [1,2]. In addition, diffusion tensor imaging (DTI), which is a useful technique for estimating myelination *in vivo* [3], showed decreased anisotropy in various regions of white matter in schizophrenic patients relative to normal controls. These regions included the prefrontal cortex, splenium, and cingulum, all of which are considered related to the pathophysiology of schizophrenia [4,5]. More recently, several groups reported decreased expression of myelin and oligodendrocyte-related genes in patients with schizophrenia using gene microarrays and quantitative polymerase chain reaction analysis [6,7]. In these studies, a highly significant decrease in transferrin expression was reported.

Transferrin is an iron transport glycoprotein synthesized primarily by hepatocytes; it is also synthesized by oligodendrocytes. Several lines of evidence indicate that brain transferrin could be involved in myelinogenesis [8]. For example, a selective culture that is essential for cultivation of neural stem cells (called the neurosphere method) contains transferrin and actually favors the outgrowth of the neurosphere [9].

Moreover, increased mRNA levels of myelin and oligodendrocyte-related genes, including *PLP, MBP, CNP, MAG, OLIG2,* and *SOX10,* have been reported in a study of transgenic mice overexpressing the complete human *transferrin* gene (*TF*) in oligodendrocytes [10]. These results suggest that transferrin can influence the maturation and differentiation of oligodendrocyte in the central nervous system [10].

The gene encoding transferrin is located on chromosome 3q22.1; linkage analyses using a genome-wide map of microsatellite DNA markers showed that the region of chromosomal 3q is implicated in the susceptibility loci for schizophrenia [11–13].

Additionally, the concentration of transferrin changes during the acute phase of schizophrenia [14,15]. Furthermore, Wong *et al.* reported that transferrin levels are reduced in schizophrenic patients relative to normal controls and can be affected by medications and disease stages [15].

Taken together, *TF* is considered a plausible candidate gene for a role in the pathophysiology of schizophrenia, although its exact role in the development of schizophrenia remains to be clarified.

Thus, in this study, we performed linkage disequilibrium (LD) analysis of *TF* followed by case–control studies examining the possible association between *transferrin* gene polymorphisms and schizophrenia in a Japanese population.

Participants and methods

Participants

The participants were 384 patients with schizophrenia [165 women, mean age ± standard deviation (SD) 56.4 ± 14.3; 219

men, 52.6±12.8] and 384 controls (162 women, 46.0±18.0; 222 men, 44.2±15.2 years).

All participants were unrelated and ethnically Japanese. A consensus diagnosis was made for each patient by experienced psychiatrists according to the *Diagnostic and Statistical Manual of Mental Disorders*, 4th ed. (American Psychiatric Association, 1994), and all available medical records and information from family members were also taken into consideration. Mentally healthy controls had no current or past contact with psychiatric services, according to unstructured interviews. This study was approved by the Ethics Committee of the Nagoya University School of Medicine, and written informed consent was obtained from all participants.

Single nucleotide polymorphism selection

TF is composed of 17 exons spanning a total length of 32.4 kb. Only one isoform of *TF* has been registered in NCBI GenBank (NM_001063_2). According to the information regarding single nucleotide polymorphisms (SNPs) of *TF* found in the dbSNP database (*http://www.ncbi.nlm.nih.gov/SNP/*) and the HapMap database (*http://www.hapmap.org/index.html.ja*: Release #19/ phase II October, 2005), the LD block was determined on the basis of the criterion $D' > 0.80$ using HAPLOVIEW software ver. 3.2 (developed by Mark Daly, URL: *http://www.broad.mit.edu/personal/jcbarret/haploview/index.php*; [16]). Haplotype tag SNPs (htSNPs) were defined as those capturing 90% of the haplotype diversity within each of the LD blocks using the same program. *TF* was constructed from a single LD block, and five SNPs (SNP1: rs8177191, SNP4: rs1799852, SNP9: rs3811647, SNP10: rs1358024, and SNP12: rs8649) were selected as htSNPs. A nonsynonymous variant SNP (SNP14: rs1049296) of *TF* was included in the analyses (Figs 1 and 2).

Single nucleotide polymorphism genotyping

Genomic DNA was extracted from the peripheral blood of all participants. rs817791 was genotyped by polymerase chain reaction-restriction fragment length polymorphism using primers (left: 5′-CCTTCTTTGGCAGCTTTTGA-3′, right: 5′-CCCTGAGGGTAAGGACCAAT-3′) and the restriction enzyme PvuII. Genotyping of the other SNPs was carried out using TaqMan assays (Applied Biosystems, Foster City, Calfornia, USA). TaqMan probes and Universal PCR Master Mix were obtained from Applied Biosystems. A 5 μl total reaction volume was used and allelic-specific

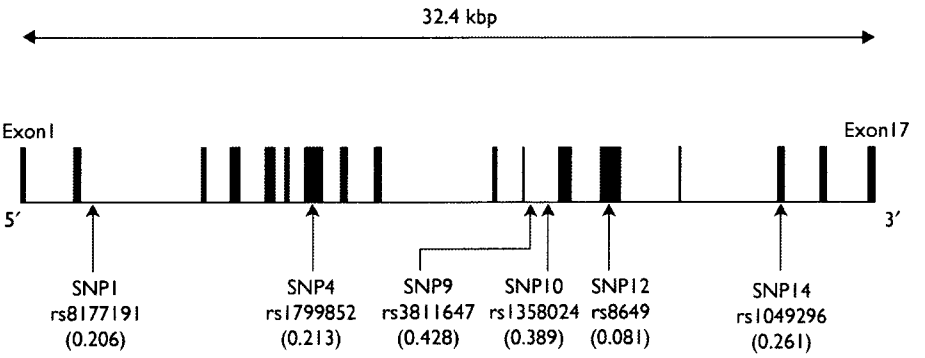

Fig. 1 Genomic structure of *TF* with single nucleotide polymorphisms (SNPs) in linkage disequilibrium mapping. Numbers under the arrows represent the six SNPs that we selected and SNPs IDs. Parenthetic numbers represent the minor allele frequencies of 384 controls.

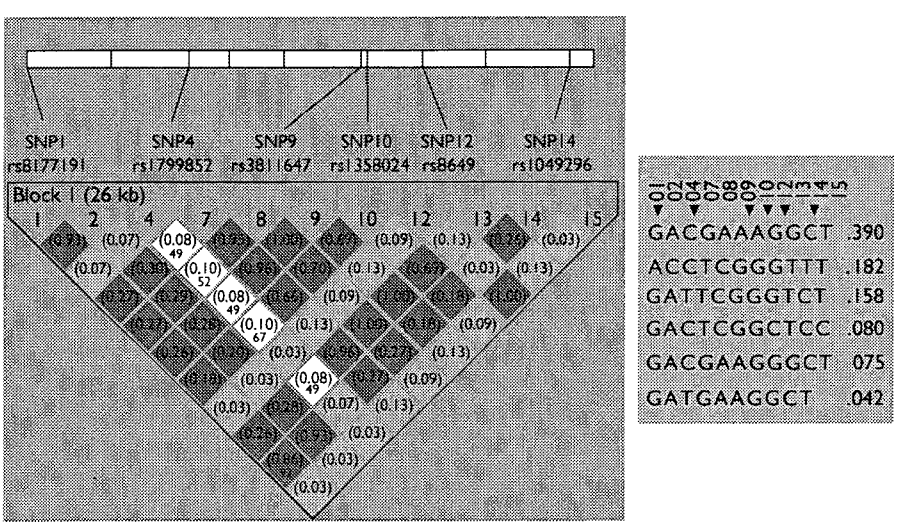

Fig. 2 (a) Linkage disequilibrium of *TF* provided by Hapmap database (release #19/ phase II October, 2005). Numbers in the box represent D' values (D' values of 1.0 are not shown). Parenthetic numbers represent the r^2 value. (b) Figure represents a pair of haplotype frequencies in *TF*.

fluorescence was measured using an ABI PRISM 7900 Sequence Detector System (Applied Biosystems).

Statistical analysis

Genotype deviation from Hardy–Weinberg equilibrium was evaluated using a χ^2 test. The associations between patients or controls with genotype and allele distributions were also evaluated using a χ^2 test. Haplotype analyses were performed using COCAPHASE version 2.403 (*http://portal.litbio.org/Registered/Option/unphased*; Dudbridge, 2003). The significance level for all statistical tests was set to 0.05. Power calculations were performed using the Genetic Power Calculator genetic statistics package (*http://pngu.mgh.harvard.edu/~purcell/gpc/*; Purcell 2001–2005).

Results

The observed genotype frequencies of the six SNPs were within the distributions expected by Hardy–Weinberg equilibrium. The LD patterns observed in our patients and controls were nearly identical to those provided by the HapMap Project.

The genotype distributions and allele frequencies of the six SNPs in the Japanese schizophrenic patients and controls are summarized in Table 1. No significant differences in genotype and allele frequencies of five htSNPs and the nonsynonymous variant SNP were observed between schizophrenic patients and controls. The distribution of haplotypic frequencies of SNP1–SNP4 (G-C) differed between the schizophrenic patients and controls (individual haplotypes, P=0.03918), but this association disappeared after correcting for multiple testing by a 10 000-iteration permutation test (P=0.1703) (Table 1).

Discussion

This study suggests that *TF* does not play an important role in the development of schizophrenia in the Japanese population, as our association analysis failed to reveal a significant association between polymorphisms of the gene and schizophrenia. As it is, however, expected that genetic risk factors for schizophrenia may differ between races or ethnicities, a replication study using different ethnic populations would be required to confirm our results.

Although highly significant decreases in the expression of *TF* in postmortem brains of schizophrenic patients have been reported, a recent study revealed that mRNA levels of brain *TF* are strongly regulated by the mRNA levels of quaking homolog, KH domain RNA binding (*QKI*) in the brains of schizophrenic patients [17]. QKI is an RNA-binding protein that regulates splicing processes subsequent to transcription [18], and is thought to regulate oligodendrocyte differentiation and maturation in the human brain [19]. It would therefore be of value to further investigate the gene–gene interactions between *TF* and *QKI* during the development of schizophrenia.

Although the levels of transferrin receptor (TfR) in the central nervous system have not been clarified, the elevation of plasma TfR in schizophrenia has been reported [20,21], and it could be hypothesized that the decrease in *TF* mRNA levels in postmortem brains observed in previous studies might reflect changes in TfR rather than sequence variations in *TF*. Therefore, it would be useful to investigate the genetic association of the *TfR* gene (*TFRC*) with schizophrenia.

Table 1 Association analyses of six SNPs in transferrin gene

SNP ID	Genotypic distribution[a]						Allelic distribution				Multi-haplotype systems[b]				
	M/M		M/m		m/m			MAF (%)							
	SCZ	CON	SCZ	CON	SCZ	CON	*P* values (genotype)	GRR	SCZ	CON	1SNP	2SNP	3SNP	4SNP	5SNP	6SNP
SNP 1(G/A) (rs817791)	248	243	122	121	12	18	0.534	1.4	19.1	20.6	0.48925					
SNP 4(C/T) (rs1799852)	263	235	105	131	16	16	0.109	1.4	17.8	21.3	0.08449	0.0363	0.19535			
SNP 9(G/A) (rs3811647)	105	129	186	180	82	74	0.242	1.33	46.9	42.8	0.1092	0.2126	0.2049	0.19542		
SNP 10(G/A) (rs1358024)	128	144	195	180	61	59	0.455	1.34	41.3	38.9	0.3431	0.05598	0.0527	0.2053	0.33119	
SNP 12(G/C) (rs8649)	317	325	62	56	4	3	0.761	1.61	9.1	8.1	0.4567	0.392	0.3907	0.2003	0.1377	0.31334
SNP 14(C/T) (rs1049296)	225	214	141	138	16	31	0.078	1.37	22.6	26.1	0.1143	0.2657				
Permutation *P* value[c]												0.1703				

[a]CON, control; GRR, genotype relative risk; M, major allele; m, minor allele; MAF, minor allele frequency; SCZ, schizophrenia; SNP, single nucleotide polymorphism.
[b]*P* values were calculated by log-likelihood ratio test (1SNP; allelewise association, 2–6SNP; global haplotypic association).
[c]An implement in the COCAPHASE.
Bold numbers represent significant *P* values.