

Glutamate Cysteine Ligase Modifier (GCLM) Subunit Gene Is Not Associated with Methamphetamine-Use Disorder or Schizophrenia in the Japanese Population

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A recent study showed a significant association between schizophrenia in European samples and the glutamate cysteine ligase modifier (GCLM) subunit gene, which is the key glutathione (GSH)-synthesizing enzyme. Since the symptoms of methamphetamine (METH)-induced psychosis are similar to those of schizophrenia, the GCLM gene is thought to be a good candidate gene for METH-use disorder or related disorders. To evaluate the association between the GCLM gene and METH-use disorder and schizophrenia, we conducted a case-control study of Japanese subjects

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(METH-use disorder, 185 cases; schizophrenia, 742 cases; and controls, 819). Four SNPs (2 SNPs from an original report and JSNP database, and 2 “tagging SNPs” from HapMap database) in the GCLM gene were examined in this association analysis; one SNP showed an association with both METH-use disorder and METH-induced psychosis. After Bonferroni’s correction for multiple testing, however, this significance disappeared. No significant association was found with schizophrenia. Our findings suggest that a common genetic variation in the GCLM gene might not contribute to the risk of METH-use disorder and schizophrenia in the Japanese population.

Key words: glutamate cysteine ligase modifier (GCLM) subunit gene; methamphetamine-use disorder; schizophrenia; linkage disequilibrium; tagging SNPs

Introduction

It has been suggested that oxidative stress and abnormality of the dopamine neural transmission system are important mechanisms in the pathophysiology of methamphetamine (METH)-use disorder and schizophrenia, and these mechanisms have been shown to be related to each other. Glutathione (GSH) plays an important role in antioxidant protection from reactive oxygen species and dopamine-induced toxicity.^{1,2} The glutamate cysteine ligase modifier (GCLM) subunit is reported to be a key enzyme in one of the GSH metabolic pathways. A recent study has shown evidence of a significant association between the GCLM gene and schizophrenia in European samples.³ The GCLM gene is located on chromosome 1p21, close to where a genome scan has shown significant linkage evidence for schizophrenia in the Japanese.⁴ Since the symptoms of methamphetamine (METH)-induced psychosis are similar to those of schizophrenia, the GCLM gene is thought to be a good candidate gene for METH-use disorder or related disorders. To evaluate the association between the GCLM gene and METH-use disorder and schizophrenia, we conducted a case-control study of Japanese samples.

We first evaluated the linkage disequilibrium (LD) structure of this gene and selected two “tagging SNPs.” Moreover, in order to reflect the LD background in the Japanese population, we included two SNPs selected from the JSNP

database and another paper. These four SNPs were then used to represent the LD properties of the gene in the Japanese population in the following association analysis.

Method

Subjects

One hundred and eighty-five patients with METH-use disorder (all patients were diagnosed with METH dependence) (148 males and 37 females, mean age \pm standard deviation [SD] 36 ± 9.4 years, 29 ± 5.68 years), 742 patients with schizophrenia (397 males and 345 females; 41.8 ± 15.7 years), and 819 healthy controls (364 males and 455 females; 37.6 ± 14.3 years) were recruited for the study. All subjects were unrelated to each other, ethnically Japanese, and lived in Japan.

The patients were diagnosed according to DSM-IV criteria, with the consensus of at least two experienced psychiatrists, on the basis of unstructured interviews and a review of medical records. All healthy controls were also psychiatrically screened through unstructured interviews. Among the subjects with METH-use disorder, 100 had a co-morbid diagnosis of METH-induced psychosis. In addition, 143 subjects with METH-use disorder also had a dependence on drugs other than METH. Subjects with METH-use disorder were excluded if they had a clinical diagnosis of psychotic disorder, mood disorder, anxiety disorder, or

eating disorder. More detailed characterizations of these subjects have been published elsewhere.⁵

The study was explained, and written informed consent was obtained from each subject. This study was approved by the Ethics Committee at Fujita Health University School of Medicine, Nagoya University Graduate School of Medicine, and each participating member of the Institute of the Japanese Genetics Initiative for Drug Abuse (JGIDA).

SNP Selection and LD Evaluation

We first consulted the HapMap database (release#21/phase II, July 2006 [www.hapmap.org]; population, Japanese in Tokyo; minor allele frequencies [MAFs] of more than 0.05) and included nine SNPs covering GCLM (5'-flanking regions including 10579bp from the initial exon and 1262bp downstream [3'] from the last exon; HapMap database contig number chr1: 94063349.94097612). Next, two "tagSNPs" (SNP1: rs2301022; SNP3: rs3170633) were selected with the criteria of r^2 threshold greater than 0.8 in "pair-wise tagging only" mode using the Tagger program (Paul de Bakker [http://www.broad.mit.edu/mpg/tagger]), an implement of the HAPLOVIEW software.⁶ Moreover, in order to reflect the LD background in the Japanese population, we included rs718875 (called SNP3 in this study) from JSNP, and ss60197536 (called SNP1 in this study) reported by Nakamura *et al.*⁷⁻⁹ Nakamura *et al.* reported that ss60197536 was responsible for the functional change.⁹ These four SNPs were selected for the following association analysis.

SNP Genotyping

We used polymerase chain reaction (PCR)-restriction fragment length polymorphism (RFLP) assay for SNP1 and TaqMan assays (Applied Biosystems, Foster City, CA, USA) for the other three SNPs. For SNP1, we obtained

the SNP information described by Nakamura *et al.* through personal communication.⁹ Detailed information on TaqMan assays, including primer sequences and reaction conditions, is available on request.

Statistical Analysis

Genotype deviation from the Hardy-Weinberg equilibrium (HWE) was evaluated by chi-square test (SAS/Genetics, release 8.2, SAS Japan Inc., Tokyo, Japan).

Marker-trait association was evaluated by chi-square test (allele and genotype-wise analyses), and the log-likelihood test (haplotype-wise analysis, where the haplotype frequencies were estimated with the expectation-maximization algorithm) (SAS/Genetics, release 8.2). To control inflation of the type I error rate, we used Bonferroni's correction. Power calculation was performed using a statistical program prepared by Ohashi *et al.*¹⁰

The significance level for all statistical tests was 0.05.

Results

Genotype frequencies of all SNPs were in HWE. The result of LD structure using 96 healthy controls can be seen Figure 1. Although a possible association of one SNP with METH-use disorder was found (SNP4: P genotype = 0.0465), this might have resulted from type I error due to multiple testing (P genotype = 0.186 after Bonferroni's correction) (Table 1). However, no association was detected between other SNPs and METH-use disorder in the Japanese population by allele/genotype-wise (Table 1) or haplotype-wise analysis ($P = 0.198$). We included an explorative analysis of clinical subgroups and gender effects for the following reasons: (1) our subjects with METH-induced psychosis were the majority of our METH subjects, so this condition would be over-represented in our samples of METH-use disorder; and (2) we had

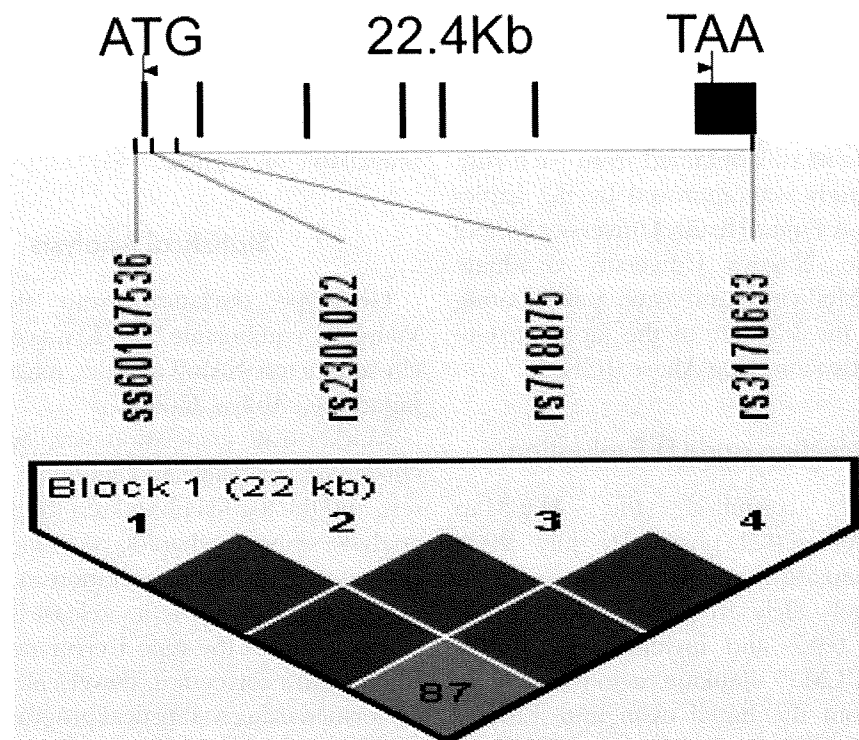


Figure 1. Linkage disequilibrium structure of the GCLM gene.

unmatched gender samples for METH-use disorder (male = 148 and female = 37). Consequently, no common results among clinical subgroups or gender were obtained. A significant association of SNP4 with METH-induced psychosis was found in a genotype-wise analysis (P genotype = 0.0447); however, this might have resulted from type I error due to multiple testing (P genotype = 0.358 after Bonferroni's correction). No association was detected between other SNPs and any subgroup or either sex (Tables 1 and 2).

We also detected no significant association between schizophrenia and controls in the allele/genotype-wise (Table 1) or haplotype analysis ($P = 0.0798$). Furthermore, we included an explorative analysis of subjects divided into gender, and detected no association in gender (Table 2).

We obtained power of more than 80% for the detection of association when we set the genotype relative risk at 1.42–1.50 in METH-use disorder and 1.25–1.30 in

schizophrenia under a multiplicative model of inheritance.

Discussion

We conducted association analysis using four SNPs, which included the original significant SNP (rs2301022) that was associated with schizophrenia, added to two SNPs from the JSNP database, and the SNP from the other original paper.⁹ We considered that these four SNPs could reflect the detailed LD background in the Japanese population. We found no association of the SNPs in the GCLM gene with METH-use disorder and schizophrenia in the Japanese population. SNP4 was associated with total METH-use disorder and METH-induced psychosis. However, these associations might have resulted from type I error because of multiple testing. Therefore, we suggest that there is no association between the GCLM gene and METH-use disorder in the Japanese population.

TABLE 1. tagSNPs and Association Analysis of the GCLM Gene

SNP ID	Phenotype ^a	MAF ^b	Genotype distribution ^c				P value ^d			Corrected P value (Genotype) ^f
			N	M/M	M/m	m/m	HWE ^e	Genotype	Allele	
SNP1 ss60197536 C > T	Controls	0.162	819	572	228	19	0.504			
	Schizophrenia	0.147	742	541	183	18	0.591	0.363	0.254	
	METH-use disorder with psychosis	0.200	185	117	62	6	0.520	0.206	0.0815	
SNP2 rs2301022 G > A	Controls	0.259	817	450	311	56	0.820			
	Schizophrenia	0.241	742	431	265	46	0.529	0.482	0.238	
	METH-use disorder with psychosis	0.243	185	108	64	13	0.412	0.675	0.534	
SNP3 rs718875 T > C	Controls	0.253	160	92	55	13	0.250	0.627	0.830	
	Schizophrenia	0.168	819	571	221	27	0.327			
	METH-use disorder with psychosis	0.145	739	542	179	18	0.485	0.239	0.0860	
SNP4 rs3170633 C > T	METH-use disorder with psychosis	0.203	185	116	63	6	0.466	0.153	0.111	
	Controls	0.197	160	101	55	4	0.271	0.156	0.209	
	Schizophrenia	0.198	819	532	250	37	0.275			
	METH-use disorder with psychosis	0.189	742	487	230	25	0.734	0.510	0.519	
		0.224	185	107	73	5	0.0686	0.0465	0.252	0.186
		0.225	160	92	64	4	0.0631	0.0447	0.268	0.358

^aWith psychosis: METH-use disorder with psychosis.

^bMAF: minor allele frequency.

^cM: major allele; m: minor allele.

^dBoldface represents significant P value.

^eHardy-Weinberg equilibrium.

^fCalculated using Bonferroni's correction.

Four of eight SNPs used in the study of Tomic *et al.*, who reported a significant association between the GCLM gene and schizophrenia in European samples, were also included in the present study.³ Our study used larger sample sizes, and the genotype frequencies of all SNPs were in HWE.

We confirmed LD for the GCLM gene at genetic differences among ethnic groups such as Han Chinese and Yoruba using SNP information from the HapMap database. Because the LD for the GCLM gene reflected the discrepancies between ethnic groups, we discussed the differences in the results that might have occurred.

A few points of caution about our present findings should be stressed. (1) For METH-

use disorder, the lack of significant associations might be due to type II error, possibly because of population stratification, unmatched gender numbers (male = 153, female = 38), clinical subgroup sample (subjects with METH-induced psychosis were the majority of our METH samples), and small sample size, as described above. (2) We did not include a mutation scan to detect rare variants with functional effects. However, it is difficult to evaluate the association of such extremely rare variants (e.g., MAF < 0.01) from the viewpoint of statistical power.

Our results thus suggest that the GCLM gene does not play a major role in METH-use disorder and schizophrenia in the Japanese population. However, the number of METH subjects

TABLE 2. Explorative Analysis of Gender and Clinical Subgroups

SNP ID	Phenotype	MAF	Genotype distribution				P value		
			N	M/M	M/m	m/m	HWE	Genotype	Allele
SNP1 ss60197536	Male controls	0.169	364	254	97	13	0.330		
	Male SCZ	0.136	397	295	96	6	0.566	0.121	0.0736
	Male	0.209	148	92	50	6	0.807	0.242	0.127
	METH-use disorder								
	With psychosis	0.193	132	85	43	4	0.605	0.427	0.375
SNP2 rs2301022	Male controls	0.260	364	200	139	25	0.899		
	Male SCZ	0.218	397	243	135	19	0.964	0.163	0.0561
	Male	0.236	148	88	50	10	0.433	0.626	0.440
	METH-use disorder								
	With psychosis	0.246	132	77	45	10	0.349	0.703	0.669
SNP3 rs718875	Male controls	0.176	364	251	98	15	0.176		
	Male SCZ	0.139	395	291	98	6	0.486	0.0623	0.0502
	Male	0.213	148	91	51	6	0.730	0.231	0.168
	METH-use disorder								
	With psychosis	0.197	132	84	44	4	0.537	0.350	0.445
SNP4 rs3170633	Male controls	0.205	364	234	111	19	0.227		
	Male SCZ	0.188	397	260	125	12	0.514	0.308	0.403
	Male	0.233	148	84	59	5	0.162	0.105	0.314
	METH-use disorder								
	With psychosis	0.227	132	76	52	4	0.162	0.133	0.440
SNP1 ss60197536	Female controls	0.157	455	318	131	6	0.0639		
	Female SCZ	0.161	345	246	87	12	0.221	0.0804	0.840
	Female	0.162	37	25	12	0	0.239	0.715	0.909
	METH-use disorder								
	With psychosis	0.196	28	17	11	0	0.196	0.432	0.436
SNP2 rs2301022	Female controls	0.258	453	250	172	31	0.848		
	Female SCZ	0.267	345	188	130	27	0.497	0.869	0.706
	Female	0.270	37	20	14	3	0.804	0.957	0.821
	METH-use disorder								
	With psychosis	0.286	28	15	10	3	0.508	0.738	0.65
SNP3 rs718875	Female controls	0.162	455	320	123	12	0.965		
	Female SCZ	0.152	344	251	81	12	0.0964	0.450	0.628
	Female	0.162	37	25	12	0	0.239	0.502	0.989
	METH-use disorder								
	With psychosis	0.196	28	17	11	0	0.196	0.284	0.493
SNP4 rs3170633	Female controls	0.193	455	298	139	18	0.723		
	Female SCZ	0.190	345	227	105	13	0.843	0.989	0.902
	Female	0.189	37	23	14	0	0.156	0.349	0.948
	METH-use disorder								
	With psychosis	0.214	28	16	12	0	0.149	0.253	0.686

used in this study was small, and even though it is difficult to find such subjects, it will be necessary to validate or replicate our association in other large-population studies.

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Conflicts of Interest

The authors declare no conflicts of interest.

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Brief Research Communication

No Association Between Prostate Apoptosis Response 4 Gene (PAWR) in Schizophrenia and Mood Disorders in a Japanese Population

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Altered dopamine D2 receptor (D2R) is hypothesized to be a susceptibility factor for major psychosis. Recent studies showed that a new intracellular protein, prostate apoptosis response 4 (Par-4), plays a critical role in D2R signaling. We conducted a genetic association analysis between Par-4 gene (PAWR) and schizophrenia and mood disorders in a Japanese population (schizophrenia: 556 cases, bipolar disorder (BP): 150 cases, major depressive disorder (MDD): 312 cases and 466 controls). Applying the recommended 'gene-based' association analysis, we selected five tagging SNPs in PAWR from the HapMap database. No significant association was obtained found with schizophrenia or MDD or BP. We found a significant association of one tagging SNP with BP in a genotype-wise analysis ($P = 0.0396$); however, this might be resulted from type I error due to multiple testing ($P = 0.158$ after SNPSpD correction). Considering the size of our sample and strategy, our results suggest that the PAWR does not play a major role in schizophrenia or mood disorders in the Japanese population.

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KEY WORDS: schizophrenia; mood disorders; Par-4; linkage disequilibrium; tagging SNP

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INTRODUCTION

An abnormal dopamine neurotransmission system is thought to contribute to the pathophysiology of psychiatric disorders such as schizophrenia and mood disorders, since the main target of most antipsychotics is dopamine D2 receptor (D2R) blockade. To date, several investigations have suggested that cellular mechanisms and signaling cascades related to D2R blockade may be associated with schizophrenia; for example AKT/Glycogen Synthase Kinase 3 (GSK3) in particular is an attractive candidate molecule for schizophrenia, since it has been reported from dopamine transporter (DAT) knock-out (KO) mice studies that the AKT/GSK3 cascade mediates dopamine-dependent behaviors through dopamine D2Rs [Beaulieu et al., 2004].

A more recent study showed that prostate apoptosis response 4 (Par-4) is also an attractive molecule from the viewpoint of psychiatric disorders [Park et al., 2005]. In this report, Par-4 was shown to interact with the D2R at the calmodulin bindings motif in the third cytoplasmic loop of the D2R, and formation of a Par-4/D2R complex was necessary to maintain an inhibitory tone on dopamine mediated cyclic AMP signaling generated by D2R in the low calcium condition [Park et al., 2005]. Moreover, Par-4 mutant mice showed depressive-like behaviors such as amotivation and anhedonia. These depressive-like symptoms of these mutant mice were reversed by antidepressants, indicating that Par-4 is also a good candidate molecule for mood disorders [Park et al., 2005].

The Par-4 gene (PAWR; OMIM *601936, 8 exons in this genomic region spanning 99.85 kb) is located on 12q21, which was shown to be a susceptibility region for schizophrenia [Wilcox et al., 2002], bipolar disorder (BP) [Craddock et al., 1994; Ewald et al., 2002], and major depressive disorder (MDD) [Craddock et al., 1994; Abkevich et al., 2003; McGuffin et al., 2005]. Considering all the above, Par-4 gene (PAWR) is a good candidate gene not only for schizophrenia but also for mood disorders (BP and MDD).

In this study, we aim to examine the genetic association between PAWR and schizophrenia or mood disorders in the Japanese population. To address this issue, we applied the recently recommended strategy of 'gene-based' association analysis [Neale and Sham, 2004]. We conducted a case-control association analysis using relatively large samples by selecting the 'tagging SNPs' from the HapMap database.

MATERIALS AND METHODS

Subjects

Five hundred and fifty-six patients with schizophrenia (289 males and 267 females; mean age \pm standard deviation (SD) 42.84 ± 14.58 years, 45.13 ± 14.9 years), 312 patients with MDD (155 males and 157 females: 44.49 ± 14.09 years,

50.43 ± 17.12 years), 150 patients with BP (76 males and 74 females: 93 patients with Bipolar I disorder and 57 patients with Bipolar II disorder: 46.58 ± 12.55 years, 45.05 ± 14.69 years) were recruited as case subjects, and a total of 466 healthy controls (243 males and 223 females: mean age ± SD 34.59 ± 12.58 years SD 36.16 ± 15.54 years) were recruited as control subjects. All subjects were unrelated with each other, ethnically Japanese, and lived in the central area of Japan.

The patients were diagnosed according to DSM-IV criteria with consensus of at least two experienced psychiatrists on the basis of unstructured interviews and review of medical records. All healthy controls were also psychiatrically screened based on unstructured interviews. Subjects were free of past or present major or minor mental illness.

After description of the study, written informed consent was obtained from each subject. This study was approved by the Ethics Committee at Fujita Health University School of Medicine and Nagoya University Graduate School of Medicine.

SNP Selection and LD Evaluation

We first consulted the HapMap database (release #21/phase II, July 2006, www.hapmap.org, population: Japanese Tokyo: minor allele frequencies (MAFs) of more than 0.1) and included 32 SNPs covering PAWR (5'-flanking regions including 1,990 bp from the initial exon and 1,046 bp downstream (3') from the last exon: HapMap database contig number chr12: 78483214...78590257) for an *in silico* 'tagging SNPs' selection. Then we applied the criterion of r^2 threshold greater than 0.8 in 'pair-wise tagging only' mode using the 'Tagger' program (Paul de Bakker, <http://www.broad.mit.edu/mpg/tagger>), five 'tagging (tag) SNPs' (SNP1: rs2463169, SNP2: rs2400546, SNP3: rs17005769, SNP4: rs4842318, SNP5: rs7305141) were selected for the following association analysis.

SNP Genotyping

All SNPs were genotyped by TaqMan assay (Applied Biosystems, Foster City, CA). Detailed information is available on request.

Statistical Analysis

Genotype deviation from the Hardy-Weinberg equilibrium (HWE) was evaluated by chi-square test (SAS/Genetics, release 8.2, SAS Japan Inc, Tokyo, Japan).

Marker-trait association was evaluated by the χ^2 test (allele and genotype-wise analyses), and the log-likelihood test (haplotype-wise analysis, where the haplotype frequencies were estimated with the Expectation-Maximization algorithm) (SAS/Genetics, release 8.2). To control inflation of the type I error rate, we used a recently developed software program, SNPSpD, which can reflect the correlation of markers (LD) on corrected P -values [Nyholt, 2004].

Power calculation was performed using a statistical program prepared by Ohashi et al. [2001].

The significance level for all statistical tests was 0.05.

RESULT

Genotype frequencies of all SNPs were in HWE for each group (P values > 0.05). The result of LD structure from HapMap database can be seen in Supplementary Figure 1. Each LD structure of SCZ, BP, MDD, and control samples was almost same (data not shown).

No association was detected with schizophrenia or MDD in allele/genotype-wise analyses (Table I), or in haplotype-wise analysis (Global P = 0.142 in schizophrenia, 0.143 in MDD; Table II). Only SNP4 showed a significant association with BP in a genotype-wise analysis (P = 0.0396). Nevertheless, this significance may be a result of type I error due to multiple testing. We performed P -value correction by using SNPSpD program (the effective number of independent marker loci: 3.98; the experiment-wide significance threshold required to keep type I error rate at 0.05: 0.0126), and the haplotype-wise analysis showed no association with BP (Global P = 0.158).

Power analyses showed that the power was more than 80% when genotype relative risk at 1.30–1.33, 1.47–1.56, and 1.70–2.38 in schizophrenia, 1.34–1.37, 1.54–1.71, and 1.79–2.54 in MDD, 1.45–1.52, 1.79–2.11, and 2.10–3.16 in BP and under a multiplicative, dominant and recessive models of inheritance, respectively.

DISCUSSION

In this study, no association of PAWR with schizophrenia and mood disorders was found through genetic case-control study.

We included an explorative analysis of subjects divided by clinical diagnosis (except MDD) or sex, and no association was detected in any subgroup or in either sex (Supplementary Tables 1, 2, 3, 4, and 5). However, we assume that quantitative traits (e.g., negative symptoms for schizophrenia patients and severity of Hamilton Depression Scale for MDD patients) will be key features in assessing the genetic contribution of PAWR to schizophrenia or MDD, since Par-4 mutant mice showed depression-like symptoms.

Psychiatric disorders are widely known as complex diseases which are characterized by the contribution of multiple susceptibility genes and environmental factors. Even though our results did not support the contribution of PAWR to such disorders, novel candidate molecules or genes related to D2R signaling should be examined. In this regard, our colleagues examined the association of AKT1/GSK3 β (and β -Arrestin2) with schizophrenia, and found that only AKT1 was associated with schizophrenia [Ikeda et al., 2004, 2005, 2007]. From these observations, we speculate that it may not be appropriate only to analyze the single-gene association for detecting susceptibility factors. Thus it will be necessary to account the gene-gene and gene-environmental interactions to obtain conclusive results.

A few points of caution about the present findings should be stressed. First, the lack of association may be due to biased samples, such as unmatched aged samples, or small sample size, especially BP samples. On the average level, the controls are much younger than the patients. This means that a number of young controls may go on to develop these disorders, the most likely MDD, since incidence of major depression is high as 5% or more. Second, we did not include a mutation scan to detect rare variants with functional effects. However, it is difficult to evaluate the association of such extremely rare variants (e.g., MAF < 0.01) from the viewpoint of power. Third, although Japanese population is considered to be homogeneous, small stratification may affect this negative finding. Replication study or family based association approach will be required for conclusive results.

Our results were robust in terms of study design, high powers of sample size, and conservative correction of multiple testing. Thus, we conclude that PAWR is unlikely to be a susceptibility gene for schizophrenia and mood disorders considering the common disease-common variant hypothesis. However, further investigations will be necessary for conclusive results.

TABLE I. tagSNPs and Association Analysis of PAWR

SNP ID	Phenotype	Position	MAF (%)	Genotype distribution ^a				P-value ^b			Corrected P-value (genotype) ^c
				N	M/M	M/m	m/m	HWE ^d	Allele	Genotype	
SNP1 rs2463169 (G > A)	Schizophrenia	0	0.300	277	224	55	0.329	0.872	0.517	0.158	
	Bipolar disorder		80	58	12	0.744	0.317	0.502			
	Major depressive disorder		138	146	28	0.225	0.402	0.608			
SNP2 rs2400546 (A > T)	Controls	14682	0.304	223	203	40	0.516	0.786	0.474		
	Schizophrenia		328	189	39	0.106	0.408	0.717			
	Bipolar disorder		90	50	10	0.408	0.453	0.656			
SNP3 rs17005769 (G > A)	Major depressive disorder	23921	0.252	172	123	17	0.408	0.453	0.656		
	Controls		466	272	169	25	0.851	0.254	0.414		
	Schizophrenia		556	321	194	41	0.125	0.121	0.205		
SNP4 rs4842318 (C > T)	Bipolar disorder	34881	0.317	68	69	13	0.441	0.121	0.205		
	Major depressive disorder		176	121	15	0.313	0.210	0.261			
	Controls		250	180	36	0.650	0.822	0.161			
SNP5 rs7305141 (A > G)	Schizophrenia	63475	0.293	282	222	52	0.388	0.128	0.0396		
	Bipolar disorder		89	49	12	0.167	0.726	0.439			
	Major depressive disorder		162	125	25	0.897	0.986	0.237			
	Controls		0.280	228	207	31	0.0777	0.0556	0.163		
	Schizophrenia		205	247	104	0.168	0.0962	0.205			
	Bipolar disorder		39	83	28	0.372	0.0855				
	Major depressive disorder		0.365	122	152	38	0.372				
	Controls		466	160	231	75	0.581				

^aM, major allele; m, minor allele.

^bBold represents significant P-value.

^cCalculated using SNPSpD software.

^dHardy-Weinberg equilibrium.

TABLE II. Individual and Global Haplotype-Wise Analyses of PAWR

Marker	Phenotype	Haplotype frequency	P-value
GAGTA	Schizophrenia	0.286	0.572
	Bipolar disorder	0.237	0.109
	Major depressive disorder	0.267	0.771
	Controls	0.273	
GAACG	Schizophrenia	0.231	0.078
	Bipolar disorder	0.302	0.229
	Major depressive disorder	0.231	0.095
	Controls	0.266	
ATGCA	Schizophrenia	0.226	0.910
	Bipolar disorder	0.229	0.994
	Major depressive disorder	0.240	0.577
	Controls	0.228	
GAGCG	Schizophrenia	0.149	0.247
	Bipolar disorder	0.154	0.423
	Major depressive disorder	0.125	0.744
	Controls	0.131	
AAGCA	Schizophrenia	0.0592	0.621
	Bipolar disorder	0.0399	0.0720
	Major depressive disorder	0.0681	0.752
	Controls	0.0644	
		Global P-value	
Schizophrenia			0.142
Bipolar disorder			0.158
Major depressive disorder			0.143

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Up-Regulation of *ADM* and *SEPX1* in the Lymphoblastoid Cells of Patients in Monozygotic Twins Discordant for Schizophrenia

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The contribution of genetic factors to schizophrenia is well established and recent studies have indicated several strong candidate genes. However, the pathophysiology of schizophrenia has not been totally elucidated yet. To date, studies of monozygotic twins discordant for schizophrenia have provided insight into the pathophysiology of this illness; this type of study can exclude inter-individual variability and confounding factors such as effects of drugs. In this study we used DNA microarray analysis to examine the mRNA expression patterns in the lymphoblastoid (LB) cells derived from two pairs of monozygotic twins discordant for schizophrenia. From five independent replicates for each pair of twins, we selected five genes, which included *adrenomedullin* (*ADM*) and *selenoprotein X1* (*SEPX1*), as significantly changed in both twins with schizophrenia. Interestingly, *ADM* was previously reported to be up-regulated in both the LB cells and plasma of schizophrenic patients, and *SEPX1* was included in the list of genes up-regulated in the peripheral blood cells of schizophrenia patients by microarray analysis. Then, we performed a genetic association study of schizophrenia in the Japanese population and examined the copy number variations, but observed no association. These findings suggest the possible role of *ADM* and *SEPX1* as biomarkers of schizophrenia. The results also support the usefulness of gene expres-

sion analysis in LB cells of monozygotic twins discordant for an illness. © 2007 Wiley-Liss, Inc.

KEY WORDS: adrenomedullin; selenoprotein; DNA microarray; gene expression; genetic association study

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INTRODUCTION

Genetic factors in schizophrenia have been shown by family, twin, and adoption studies. A higher concordance rate of schizophrenia in monozygotic twins (41–79%) compared with that in dizygotic twins (0–14%) especially supports the contribution of genetic factors in schizophrenia [Shih et al., 2004]. As the risk genes for schizophrenia, a balanced translocation disrupting disrupted schizophrenia-1 (*DISC1*) [Millar et al., 2000] and a chromosomal deletion at 22q11 [Bassett and Chow, 1999] are well established. As common variants associated with schizophrenia, dystrobrevin-binding protein 1 (*DTNBP1*) [Straub et al., 2002] and neuregulin 1 (*NRG1*) [Stefansson et al., 2002], which were identified from linkage analysis, were reported. However, the association of *DTNBP1* haplotype with schizophrenia is not consistent among studies [Mutsuddi et al., 2006]. Further studies to identify the molecular pathology of this illness are needed.

In addition to the traditional genetic approaches, an additional strategy to identify the genetic basis of endophenotypes of schizophrenia is becoming popular. In this approach, endophenotypes, measurable biological variables associated with genetic risk of schizophrenia, are first identified; then their genetic basis is studied [Braff et al., 2007]. Many established endophenotypes, such as eye tracking abnormality [Holzman et al., 1977], ventricular enlargement [Reveley et al.,

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1982], reduced hippocampal volume [Suddath et al., 1990], hypofrontality [Berman et al., 1992], and neuropsychological measures [Goldberg et al., 1995], were validated by the study of monozygotic twins discordant for schizophrenia.

In an attempt to identify molecular endophenotypes, biochemical differences in blood between the monozygotic twins discordant for schizophrenia have been investigated. These studies showed some differences between twins: plasma haptoglobin levels [Vander Putten et al., 1996], DNA methylation status [Tsujita et al., 1998; Petronis et al., 2003], soluble interleukin-2 receptors (SIL-2Rs) [Rapaport et al., 1993], mRNA expression level of a certain transcript [Friedhoff et al., 1995], retrovirus [Deb-Rinker et al., 1999], catecholamine levels [Walker et al., 2002], DNA stability [Nguyen et al., 2003], and lipid metabolism [Tsang et al., 2006]. On the other hand, no difference was found for viral nucleic acids [Sierra-Honigmann et al., 1995], platelet monoamine oxidase activity [Reveley et al., 1983], and genomic sequences [Polymeropoulos et al., 1993; Vincent et al., 1998; McDonald et al., 2003]. If a robust difference between discordant twins is well validated, such a finding will become a clue to identify the cause of this difficult illness [Kato et al., 2005a].

To identify the genes differentially expressed between the twins, one may use peripheral blood cells. However, this method is hampered by the fact that most of the patients are under treatment with drugs such as antipsychotics, which potentially affect the gene expression patterns. One possible method to avoid these confounding factors is to use the lymphoblastoid (LB) cells. Gene expression patterns in LB cells can be assessed with minimum inter-individual variability [Cheung et al., 2003], and the effect of drugs may be avoided or reduced by culturing the cells.

We previously performed DNA microarray analysis and examined the mRNA expression pattern using LB cells of monozygotic twins discordant for bipolar disorder. On the basis of our findings, we suggested the possible contribution of the endoplasmic reticulum stress response pathway to the pathophysiology of the illness [Kakiuchi et al., 2003]. Recently, Matigian et al. [2007] also performed DNA microarray analysis in three pairs of monozygotic twins discordant for bipolar disorder and found that genes related to the WNT signaling pathway were altered in patients. Several other groups have also applied the similar strategy to other illnesses such as autism and rheumatoid arthritis [Haas et al., 2006; Hu et al., 2006].

In this study, we used DNA microarray analysis to examine the mRNA expression pattern in the cells of two pairs of monozygotic twins discordant for schizophrenia. Because one of the problems in this strategy is lack of statistical analysis due to small sample size, we performed five independent experiments for each pair of twins. The expression of five genes commonly was shown to be altered in both of the twins, and two genes survived after the exclusion of three immunoglobulin-related genes. Interestingly, both of the final genes, *adrenomedullin* (*ADM*) and *selenoprotein X1* (*SEPX1*), had been reported to be up-regulated in the cells or plasma of schizophrenic patients. We further tried to identify the genetic basis of up-regulation of *ADM* and *SEPX1* levels in schizophrenia by a case-control association analysis of schizophrenia in the Japanese population. Because copy number variation (CNV) was reported to exist around these loci, CNV was also examined.

MATERIALS AND METHODS

Subjects

For the DNA microarray analysis, two pairs of monozygotic twins discordant for schizophrenia (SZ twins) were recruited.

The SZ twins A were 54-year-old males, and SZ twins B were 24-year-old females, who were previously reported elsewhere [Kunugi et al., 2003].

The SZ twins A were diagnosed by the consensus of two senior psychiatrists after independent unstructured interviews. Their family history was obtained from interviews of the twins. They had two healthy sisters, and their parents did not have major mental disorders. The affected twin of this pair (SZ-twin-A1) graduated from a university and worked as an office worker for 2 years. At age 25, he developed disorganized behavior and thought, accompanied by excitation. He also had non-systematic delusion of persecution and auditory hallucination. He was hospitalized in a psychiatric ward for 3 months. After the first episode, he was admitted to psychiatric hospitals 13 times. He began to develop negative symptoms and changed jobs several times because of interpersonal problems. He married at age 32, but divorced 1 year later. After that, he could not continue to work and lived alone, supported by social welfare. His diagnosis according to the International Classification of Diseases, Revision 10 (ICD-10) was schizophrenia, disorganized type. He was also diagnosed to have diabetes mellitus. He had been treated with 150 mg of clozapamine hydrochloride, a typical antipsychotic, and 3 mg of trihexyphenidyl hydrochloride, as an antiparkinsonian drug. It is not known whether his diabetes is a side effect of these drugs. His co-twin had been working at a company for 30 years and had been married. He was not diagnosed to have any major mental disorders or personality disorders. He did not have diabetes.

The proband of SZ twins B was diagnosed by the consensus of two senior psychiatrists after independent unstructured interviews. The diagnosis of the proband according to the Diagnostic and Statistical Manual of Mental Disorders, Fourth Edition (DSM-IV; American Psychiatric Association) was schizophrenia. Her co-twin was interviewed with the use of the schedule for affective disorders and schizophrenia (SADS), which revealed no current or past history of affective disorders or psychotic disorders. Their mother was interviewed and found to be healthy. Their father was also healthy, according to the available information. The symptoms of the proband is minutely described elsewhere [Kunugi et al., 2003]. In brief, the proband's symptoms began around the age of 15, with delusion of persecution. After that, she developed auditory hallucination and negative symptoms.

For the case-control association study, the genomic DNA derived from peripheral blood cells of 223 patients with schizophrenia (45.7 ± 14.9 years old, 129 males and 94 females) and 364 controls (50.4 ± 12.5 years old, 184 males and 180 females) in the Japanese population were analyzed. They were diagnosed according to the DSM-IV criteria. Controls were selected from students, nurses, office workers, and doctors in participating institutes, and their friends. A senior psychiatrist interviewed the controls and found no major mental disorders. Only a subset of the controls were interviewed with the use of a structured interview, the mini-international neuropsychiatric interview (M.I.N.I.) [Sheehan et al., 1997]. In the Japanese population, no significant population stratification has been repeatedly reported in several studies [Kakiuchi et al., 2003; Arinami et al., 2005; Shimizu et al., 2006].

For the quantitative genomic polymerase chain reaction (PCR), we used genomic DNA derived from LB cells of the two pairs of discordant SZ twins, 46 Japanese unrelated schizophrenia patients (38.6 ± 14.6 years old, 18 males and 28 females), and 11 controls (56.3 ± 11.0 years old, 8 males and 3 females), and 13 schizophrenia patients (55.0 ± 9.9 years old, 9 males and 4 females) obtained from NIMH Genetics Initiative Pedigrees. Written informed consent was obtained from all subjects. The ethics committees of the Brain Science

Institute (RIKEN) and participating institutes approved the study.

Cell Culture

The lymphocytes derived from peripheral blood were transformed by Epstein-Barr (EB) virus and cultured with the use of standard techniques as described before [Kato et al., 2002]. For mRNA quantification by DNA microarray analysis, we extracted the RNA from frozen cells, and thawed and recultured the cells. The culture of the cells and mRNA extraction were performed independently five times for each pair of twins.

DNA Microarray

DNA microarray experiments were performed as described previously with the use of an Affymetrix HU133A chip (Affymetrix, Santa Clara, CA) [Kakiuchi et al., 2006]. We used 5 μ g of total RNA for reverse-transcription into cDNA, and biotin-labeled cRNA was synthesized from the cDNA. After testing the integrity of the samples by the Test2Chip (Affymetrix), fragmented cRNA was applied to the HU133A chip. The hybridization signal on the chip was scanned and subjected to image analysis (Affymetrix).

Analysis of DNA Microarray Data

The microarray raw data were processed by MAS5.0 (Affymetrix) and robust multiarray average (RMA) methods [Irizarry et al., 2003], and analyzed with the use of GeneSpring software (SiliconGenetics, Redwood, CA). Data were normalized by the median value. Genes expressed differently in each pair of twins were selected by the following criteria: (1) the genes were called as present in all samples (five samples of affected twin and five samples of control co-twin); (2) both the parametric test and the non-parametric test showed a significant difference ($P < 0.05$) between the five cultures in a patient and five cultures in the co-twin by both normalization methods (MAS5.0 and RMA). Then, the genes commonly changed to the same trend in both SZ twins A and SZ twins B according to these four statistical comparisons: MAS5 and RMA, parametric and non-parametric.

Genetic Association Studies

We selected five SNPs (rs7944706, rs6484148, rs6484147, rs4597056, rs726102) for *ADM* according to the linkage disequilibrium (LD) map database on SNPbrowserTM (Applied Biosystems, Foster City, CA). Although a previous report in the Japanese population hypothesized a possible role of dinucleotide repeat in the 4 kb downstream of *ADM* in the pathophysiology of hypertension, this microsatellite marker was not associated with plasma ADM concentration [Ishimitsu et al., 2001]; thus, this marker was not selected for the analysis. We selected three SNPs (rs9928312, rs9934331, rs1003904) for *SEPX1*, because their TaqMan probes were commercially available and they are polymorphic in Japanese according to the LD map database on HapMap projects accessed with the SNPbrowserTM software. We performed genotyping by TaqMan probes and ABI7900HT according to the protocol recommended by the manufacturer (Applied Biosystems). Assessment of LD patterns by the standardized disequilibrium coefficient (D') and squared correlation coefficient (r^2), and analysis of haplotypic distribution, and frequencies were performed with the use of the COCAPHASE programs (<http://portal.litbio.org/Registered/Option/unphased.html>). Global significance was calculated by the random permutation test (10,000 times).

Quantification of Genome Copy Number

The copy number of *ADM* and *SEPX1* was analyzed by the real-time PCR method with the use of SYBR/GREEN dye

(Applied Biosystems) as described elsewhere [Kato et al., 2005b]. *MLC1* (megalencephalic leukoencephalopathy with subcortical cysts gene 1) was used as a single copy control gene and the copy number of *ADM* was calculated as a relative ratio to *MLC1*. A minimum of three probes for *ADM* was used. For quality control, a gene on the X chromosome [phosphofructo-2-kinase (*PF2K*)] was also examined by SYBR/GREEN dye, and separation between males and females was confirmed. Sequences of primers and probes for these analyses will be provided upon request.

RESULTS

Microarray Analysis in the Cells of Monozygotic Twins Discordant for Schizophrenia

By the criteria described above, five genes were identified (Table I). Among the up-regulated genes in schizophrenia, two genes (GenBank accession nos. L06101 and Z00008) were immunoglobulin-related genes, and *CD200* (GenBank accession no. AF063591) was also a member of the immunoglobulin superfamily (OMIM 155970). This result possibly reflects transformation of a subset of B-cells by the EB virus rather than a difference in disease state. Surprisingly, both of the finally listed genes [*ADM* (GenBank accession no. NM_001124) and *SEPX1* (GenBank accession no. NM_016332)] have been reported to be altered in schizophrenia. The mRNA expression of *ADM* was reported to be up-regulated in the LB cells derived from schizophrenia patients, and the plasma ADM level was significantly higher in schizophrenic patients than in controls [Zoroglu et al., 2002; Huang et al., 2004; Yilmaz et al., 2007]. *SEPX1* was included in the list of genes up-regulated in the peripheral blood cells of schizophrenia by microarray analysis [Glatt et al., 2005]. Interestingly, the expressions of both genes were up-regulated in all the studies, which was the same trend shown in this study. These results suggested that *ADM* and *SEPX1* were strong candidate genes for schizophrenia.

Association Analysis of *ADM* and *SEPX1* in Schizophrenia

If up-regulation of *ADM* and *SEPX1* is a risk factor for schizophrenia, genetic variations of these genes may contribute to the illness. Thus, we also performed association analysis of *ADM* and *SEPX1* in schizophrenia in the Japanese population. We examined the genotype of five SNPs for *ADM* and three SNPs for *SEPX1*. LD patterns for *ADM* and *SEPX1*, as measured by D' and r^2 , are shown in Figure 1. No significant association was observed in single SNPs (Table II) and haplotypes (Table III) for *ADM*, and in single SNPs for *SEPX1* (Table II).

Quantification of Genome Copy Number

In addition to sequence variations, CNVs may also contribute to the up-regulation of *ADM* and *SEPX1*. Indeed, CNVs were reported for the loci of both genes [*ADM* (RP11-79E12) and *SEPX1* (RP11-451K7 and Variation_5329), <http://projects.tcag.ca/variation/>]. The CNV may cause altered mRNA expression and may confound the results of association analysis. Thus, we quantified the copy number of *ADM* and *SEPX1* genes by the real-time PCR method in two pairs of discordant SZ twins, 46 Japanese unrelated schizophrenia patients, and 11 controls, and from genetic information for 13 schizophrenia patients obtained from NIMH Genetics Initiative Pedigrees. However, we observed no loss or gain of the genome in the tested loci (data not shown).

TABLE I. The Result of DNA Microarray Analysis in the Lymphoblastoid Cells of Monozygotic Twins Discordant for Schizophrenia

Probe ID	Genbank	Symbol	SZ twin1						SZ twin2								
			MAS			RMA			MAS			RMA					
			FC	P(P)	P(non-P)	FC	P(P)	P(non-P)	FC	P(P)	P(non-P)	FC	P(P)	P(non-P)			
Up-regulation																	
211641_x_at	L06101		1.428	0.042	0.028	1.273	0.022	0.009	1.380	0.030	0.028	1.277	0.011	0.016	0.016	0.016	0.016
217977_at	NM_016332	SEPX1	1.183	0.015	0.047	1.175	0.012	0.016	1.124	0.044	0.028	1.129	0.011	0.028	0.028	0.028	0.028
202912_at	NM_001124	ADM	1.622	0.011	0.028	1.564	0.005	0.009	1.966	0.002	0.009	1.922	0.004	0.009	0.009	0.009	0.009
216517_at	Z00008		1.684	0.022	0.028	1.683	0.038	0.028	2.076	0.000	0.009	1.839	0.001	0.009	0.001	0.009	0.009
Down-regulation																	
209583_s_at	AF063591	CD200	0.755	0.016	0.009	0.750	0.019	0.009	0.603	0.003	0.016	0.704	0.005	0.016	0.005	0.016	0.016

FC, fold change; P(P(non-P)), P-value calculated by parametric/non-parametric test using GeneSpring software.

DISCUSSION

In this study, we demonstrated that mRNA expressions of *ADM* and *SEPX1* were up-regulated in the LB cells of the two patients with schizophrenia compared with their healthy co-twins. This observation is consistent with the previous reports examined in unrelated patients and controls. Genetic association studies of *ADM* and *SEPX1* for schizophrenia in the Japanese population, however, did not support the association of SNPs in these genes with schizophrenia. Further, we did not observe CNVs in these genes.

ADM is a potent vasodilator peptide consisting of 52 amino acids (OMIM103275), which was initially identified from pheochromocytoma [Kitamura et al., 1993]. *ADM* is synthesized by many tissues including the central nervous system and is known to bind to calcitonin receptor-like receptor. The reported roles of *ADM* are variable, such as dilation of blood vessels and increase in urine output. *ADM* is also abundantly expressed in the central nervous system, especially in the thalamus, hypothalamus, and pituitary gland, and it regulates neuroendocrine response to stress [Taylor and Samson, 2004]. Intracerebroventricular administration of *ADM* is known to affect water intake and salt appetite. A probably reactive increase of *ADM* in plasma is reported in some diseases such as heart failure, renal diseases, septic shock, and diabetes mellitus [Beltowski and Jamroz, 2004]. This increased level in plasma was first reported in patients with schizophrenia [Zoroglu et al., 2002]. This observation might reflect reactive up-regulation associated with some somatic condition associated with schizophrenia. However, elevated mRNA levels also were reported in LB cells of schizophrenia patients [Huang et al., 2004], which suggested that increase of *ADM* is intrinsic rather than reactive. In this study, *ADM* mRNA level was increased in the affected co-twins. Thus, intrinsic increase of *ADM* may be related to the pathophysiology of schizophrenia.

SEPX1 is one of the selenoproteins, which includes selenocysteine, and is abundant in liver, leucocytes, and pancreas (OMIM 606216). The function of *SEPX1* has not been clarified; however, interestingly, selenium-binding protein1 (*SELENBP1*), which also binds to selenium, was demonstrated to be up-regulated in both the brain and the peripheral blood leukocytes in patients with schizophrenia, and was suggested to be a candidate biomarker of schizophrenia [Glatt et al., 2005]. In the list of genes up-regulated in peripheral blood cells in this report, *SEPX1* was also included. In the present study, *SEPX1* mRNA level was also increased in the affected co-twins. Thus, the up-regulation of *SEPX1* may play a role in the pathophysiology of schizophrenia. Geographical analysis showed that low selenium in soil and food might be associated with schizophrenia [Brown, 1994]. At deficiency selenium is preferentially retained in the brain compared with other organs, and several studies have shown that selenium deficiency is associated with mood [Benton, 2002]. A possible role of selenium transport has been proposed in schizophrenia [Berry, 1993]. Thus, the roles of selenium metabolism in pathophysiology of schizophrenia may merit further study.

Although linkage with schizophrenia and presence of CNVs around the *ADM* and *SEPX1* loci [Yamada et al., 2004; Moon et al., 2006; Redon et al., 2006] prompted us to perform an association study, no association was found. This result suggests that up-regulation of *ADM* and *SEPX1* might be a phenomenon secondary to schizophrenia. However, in the association study, we studied only 223 schizophrenic patients and 364 control subjects. The number of the subjects and the number of SNPs examined are not large enough to totally exclude a possible association between schizophrenia and the SNPs of *SEPX1* and *ADM*. In addition, the result should be treated with caution, because there was a significant difference in gender between patients with schizophrenia and controls ($P < 0.05$).

TABLE II. The Result of Case-Control Studies in Japanese Population

		Genotype			HWE	P-value	Allele		P-value
ADM									
rs7944706		A/A	A/G	G/G			A	G	
	CT	50	176	138	0.606		276	452	
rs6484148	SZ	35	102	86	0.604	0.748	172	274	0.823
		C/C	C/T	T/T			C	T	
rs6484147	CT	43	166	155	0.887		252	476	
	SZ	25	84	114	0.121	0.117	134	312	0.106
rs4597056		C/C	C/T	T/T			C	T	
	CT	43	166	155	0.887		252	476	
rs726102	SZ	25	84	114	0.121	0.117	134	312	0.106
		C/C	C/T	T/T			C	T	
rs4597056	CT	157	163	44	0.865		477	251	
	SZ	114	84	25	0.121	0.160	312	134	0.116
rs726102		A/A	A/G	G/G			A	G	
	CT	42	165	157	0.892		249	479	
	SZ	25	84	114	0.121	0.147	134	312	0.140
SEPX1									
rs9928312		A/A	A/G	G/G			A	G	
	CT	45	175	144	0.464		265	463	
rs9934331	SZ	27	102	94	0.934	0.820	156	290	0.622
		C/C	C/G	G/G			C	G	
rs1003904	CT	103	176	85	0.559		382	346	
	SZ	61	109	53	0.752	0.969	231	215	0.821
rs1003904		A/A	A/G	G/G			A	G	
	CT	158	150	56	0.044		466	262	
	SZ	78	105	40	0.653	0.129	261	185	0.060

CT, control; SZ, schizophrenia; HWE, Hardy-Weiberg equilibrium. P values are calculated by Fisher's exact test.

With regard to endophenotypes of schizophrenia, mainly psychophysiological, neurocognitive, and neuroimaging findings have been proposed [Gottesman and Gould, 2003]. Relatively few studies focused on blood analysis in schizophrenia. Altered mRNA levels in LB cells were reported for *ADM* [Huang et al., 2004] and *PDLIM5* [Iwamoto et al., 2004]. Alterations in peripheral blood leukocytes mRNA were reported for *SELENBP1* and other candidate genes [Glatt et al., 2005], mitochondria-related transcripts [Whatley et al., 1998; Mehler-Wex et al., 2006], dopamine receptors [Ilani et al., 2001; Kwak et al., 2001; Zvara et al., 2005; Boneberg et al., 2006], alpha 7-nicotinic acetylcholine receptor subunit (*CHRNA7*) [Peri et al., 2006], and transforming growth factor beta receptor II (*TGFBR2*) [Numata et al., 2007]. Although none of these candidate mRNA markers in blood cells has been established, it is promising that two genes detected in this study have already been reported in the literature. *ADM* and *SEPX1* are a promising target of further research of biomarkers of schizophrenia.

After our previous report of gene expression analysis in monozygotic twins discordant for bipolar disorder [Kakiuchi et al., 2003], the same approach was used by other investiga-

tors [Haas et al., 2006; Hu et al., 2006; Matigian et al., 2007] or different tissues [Zhou et al., 2005; Cutting and Snowden, 2006; Sarkijarvi et al., 2006]. The present results that two previously reported genes were identified in the twins supported the validity of this methodology. It has been difficult to apply statistical analysis to a limited number of twin samples. Thus, in this study, we performed five independent experiments for each pair of twins. Although it is difficult to prove the validity of this method, it is possible that this extensive analysis enabled the successful selection of these two genes.

In this study, the two pairs of twins discordant for schizophrenia did not have other family history. Thus, the dysregulation of genes in the affected twin is not due to a heritable factor such as a genetic polymorphism, but rather to some environmental or epigenetic effect. Thus, lack of association of the two genes with schizophrenia may be reasonable.

Although we focused on *ADM* and *SEPX1* in this study, the change in *CD200* might also be potentially interesting, because several studies reported that the immune system in schizophrenics may be involved in its susceptibility [Nawa and Takei, 2006]. Moreover, *CD200* has a unique expression pattern that

TABLE III. Haplotype Analysis of ADM in Japanese SZ Samples

Haplotype	SZ	CT	χ^2	P-value	Global P-value
ADM					
A-T-T-C-G	168 (0.380)	270 (0.381)	0.00184	0.965	
G-C-C-T-A	132 (0.298)	238 (0.336)	1.76	0.184	
G-T-T-C-G	142 (0.321)	200 (0.282)	1.94	0.162	0.345

SZ, schizophrenia; CT, control. Global P-value was calculated by a random permutation test (10,000 times) with the use of COCAPHASE program. Only haplotypes that were verified at least once were analyzed.

A

		D'				
ADM		rs7944706	rs6484148	rs6484147	rs4597056	rs726102
r ²	rs7944706		1	1	1	1
	rs6484148	0.3172		1	1	1
	rs6484147	0.3172	1		1	1
	rs4597056	0.3093	1	1		1
	rs726102	0.3114	1	1	1	

B

		D'		
SEPX1		rs9928312	rs9934331	rs1003904
r ²	rs9928312		1	0.4592
	rs9934331	0.6233		1
	rs1003904	0.2071	0.5721	

Fig. 1. Intermarker linkage disequilibrium pattern for *ADM* (A) and *SEPX1* (B). The standardized disequilibrium coefficient (D') and squared correlation coefficient (r²) calculated by the COCAPHASE program are shown for Japanese control samples.

is expressed on B-cells and neurons [Wright et al., 2001]. *CD200* is expressed in developing neuronal cell bodies and axons [Morris and Beech, 1987]. Thus, *CD200* may be a promising target for further study.

In conclusion, we demonstrated the possible pathological contribution of *ADM* and *SEPX1* to schizophrenia and the usefulness of LB cells of monozygotic twins discordant for schizophrenia.

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

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Abstract

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

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

nificant association with the 5-HT receptor subtypes has been reported in a number of studies as follows.

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

Since the 5-HT_{3A} receptor has been reported to have potential anxiolytic and antipsychotic properties from animal studies, 5-HT_{3A} receptor antagonists are being explored as therapeutic agents for a variety of behavioral disorders [5]. Additionally, 5-HT_{3A} receptor gene (*HTR3*) is located on 11q23.1, where linkage with schizophrenia has been suggested in several studies [19,16]. These results suggest that *HTR3* may be related to

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