Instead, a role for the γ-aminobutyric acid (GABA) system in drug abuse is also suggested in accumulating evidence. First, the irreversible GABA-transaminase inhibitor, γ-vinyl GABA, attenuates such increase of the dopamine release in the nucleus accumbens following acute administration of METH.[8] Second, QTL mapping for acute alcohol withdrawal severity suggests that a polymorphism in the $GABA_A$ receptor γ2 subunit gene in mice is genetically correlated with this phenotype.[9] A third line of evidence involves several case–control association studies, suggesting that the human $GABA_A$ receptor γ2 subunit gene (GABRG2) is marginally associated with METH use disorder,[10] and is also associated with alcoholism comorbid with antisocial personality disorder,[11] although there are conflicting results.[12,13] Therefore, it is possible that GABRG2 affects vulnerability to substance use disorder, including METH use disorder.

On the other hand, a number of post-mortem studies have reported an altered GABA neurotransmission in schizophrenia. These studies reported that release and uptake of GABA at synaptic terminals were reduced in schizophrenic cortex[14–16] and that the activity of glutamic acid decarboxylase (GAD), the synthesizing enzyme for GABA, GAD mRNA expression, and the density of GABAergic interneurons, were reduced in the prefrontal cortex (PFC) of schizophrenics.[17–20] Although there was reportedly no significant change in overall mRNA levels for $GABA_A$ receptor subunits,[17] expression of the alternately spliced short isoform of $GABA_A$ receptor γ2 subunit, γ2S, was markedly reduced in the PFC of schizophrenics.[21] The relative over-representation of the γ2L subunit, which possesses an additional phosphorylation site within the eight amino acids inserted, should result in a functionally less active form of the receptor,[22,23] and this defective GABAergic system may be involved in the development of schizophrenia. The evidence of linkage analysis from multiple genome scans of schizophrenia within 5q31–34 where GABRG2 locates also support the involvement of this gene in the development of schizophrenia.[24–27]

Here, we explored the possible contributions of GABRG2 in both METH use disorder and schizophrenia. We systemically searched all exons and the intronic branch sites of GABRG2 for polymorphisms, and examined haplotype-based case–control association analysis with both METH use disorder and schizophrenia.

RESULTS

Our screening of 96 controls in all exons and the flanking intronic splice sites of GABRG2 revealed six SNPs, which were designated 'Asn79Ser', '315C>T', '588T>C', '922+20G>A', '1129–1482A>C', and '1230C>T'. Minor allele frequencies and a schematic graph of these SNPs are presented in Table 1 and Figure 1, respectively. Of all identified SNPs, 315C>T, 588T>C (rs211037) and 922+20G>A have been reported elsewhere.

To evaluate the linkage disequilibrium (LD) in the 96 screened samples using several widely used measures (D', Δ_2 and *P*-value), we genotyped five SNPs in GABRG2 (two SNPs (315C>T, 588T>C) of identified SNPs, two SNPs (rs2268583, rs2284780) from the dbSNP database, and one SNP (1128+99C>A) reported as *Bam*HI RFLP previously[11]). These SNPs were selected because they showed sufficient heterozygosity (a frequency of minor allele>0.1) to detect a small effect of a susceptibility gene presumed to underlie complex disorders, and they were distributed almost evenly on the entire exonic regions of the gene (Figure 1).

Estimation of LD between each pairwise SNP is presented in Table 2. These results show that the first three and the last two consecutive SNPs were in complete or nearly complete LD with each other. Therefore, we selected two SNPs (315C>T and 1128+99C>A) as representatives of these nearly complete LD regions for further case–control association analysis.

In addition to screened 96 samples, we genotyped 178 subjects with METH use disorder, 288 schizophrenics, and 288 controls in all. Two representative SNPs were in moderate LD with each other in METH use disorder ($D'=0.72$), schizophrenia ($D'=0.51$) and control subjects ($D'=0.61$). Genotypic and allelic frequencies of two SNPs in each population are summarized in Table 3. The genotypic distributions of each SNP did not significantly deviate from the Hardy–Weinberg equilibrium in either METH use disorder, schizophrenia or control subjects ($P=0.98$, 0.84 and 0.70 at 315C>T and $P=0.15$, 0.62 and 0.06 at 1128+99C>A, respectively). The distributions of each SNP did not differ significantly between each diagnostic group and controls in both allele and genotype frequencies (Table 3).

The distributions of haplotypic frequencies estimated using the expectation-maximization algorithm implemented in the Arlequin 2.0 significantly differed between METH use disorder and control subjects ($P=0.044$). In contrast, there was no significant difference in haplotypic distributions between schizophrenic and control subjects ($P=0.356$, Table 4). From examining at-risk haplotypes predisposed to METH use disorder, only two haplotypes, T–C and T–A (defined by 315C>T–1128+99C>A), were found to confer the significant susceptibility to this disorder. By applying the Bonferroni correction, this finding becomes nonsignificant for haplotype T–A (corrected $P=0.120$) and remains

Table 1 SNPs in GABRG2 and minor allele frequencies

SNP	*SNP position*	*Minor allele frequency*	*Reference*
107+740C>T	Intron 1	0.302	rs2268583
Asn79Ser	Exon 2	0.005	
315C>T	Exon 3	0.300	
588T>C	Exon 5	0.480	rs211037
922+20G>A	Intron 7	0.020	
923–466C>T	Intron 7	0.480	rs2284780
1128+99C>A	Intron 8	0.480	*Bam*HI C>A
1129–1482A>T	Intron 8	0.236	
1230C>T	Exon 9	0.005	

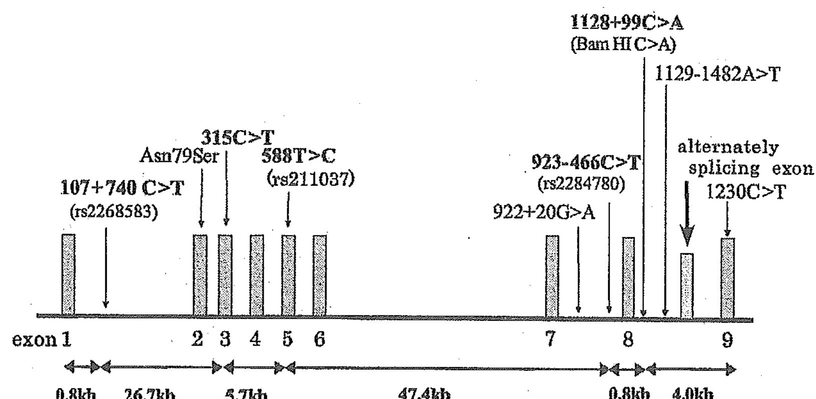

Figure 1 Schematic presentation of identified and reported GABRG2 SNPs. Solid box represents exons. The SNPs in bold type were used to evaluate LD structure.

Table 2 Pairwise linkage disequilibrium in controls

		rs2268583	*315C>T*	*D'* / Δ^2 *588T>C*	*rs2284780*	*1128+99C>A*
P-value	rs2268583		1.000 0.976	0.926 0.457	0.376 0.058	0.376 0.058
	315C>T	$<1.0\times10^{-5}$		0.962 0.482	0.608 0.141	0.608 0.141
	588T>C	$<1.0\times10^{-5}$	$<1.0\times10^{-5}$		0.643 0.315	0.643 0.315
	rs2284780	0.002	0.0002	$<1.0\times10^{-5}$		1.000 1.000
	1128+99C>A	0.002	0.0002	$<1.0\times10^{-5}$	$<1.0\times10^{-5}$	

Table 3 Genotypic and allelic distributions of the GABRG2 SNPs in patients with METH use disorder and schizophrenia *vs* controls

SNP	*Sample*	*n*	*Genotype*			*Rarer allele*	*P-value*	
			CC	*CT*	*TT*	*T*	*Genotype*	*Allele*
315C>T	METH	178	87 (49%)	75 (42%)	16 (9%)	107 (30%)	0.374	0.174
	SCZ	288	151 (52%)	116 (40%)	21 (7%)	158 (27%)	0.818	0.594
	Control	288	157 (55%)	113 (39%)	18 (6%)	149 (26%)		
			CC	*CA*	*AA*	*A*	*Genotype*	*Allele*
1128+99C>A	METH	178	56 (31%)	79 (44%)	43 (24%)	165 (46%)	0.603	0.281
	SCZ	288	64 (22%)	139 (48%)	85 (30%)	309 (54%)	0.317	0.238
	Control	288	80 (28%)	128 (44%)	80 (28%)	288 (50%)		

Table 4 Haplotypic distributions of the GABRG2 gene in patients with METH use disorder and schizophrenia *vs* controls

Sample	*Haplotypes (315C>T–1128+99C>A)*				*P-value*
	C–C	*C–A*	*T–C*	*T–A*	
METH	0.275	0.425	0.262	0.039	0.044
SCZ	0.261	0.464	0.202	0.072	0.356
Control	0.314	0.428	0.186	0.072	

significant for haplotype T–C (corrected $P=0.028$). The presumed at-risk haplotype T–C has an estimated frequency of 18.6% among controls and 26.2% among METH use disorder subjects. The estimated odds ratio of haplotype T–C was 1.55 (95% CI (1.13–2.13)).

DISCUSSION

Our results provide supportive evidence for a haplotypic association in GABRG2 with METH use disorder, but not with schizophrenia. This association suggests that the susceptibility variant for METH use disorder may lie within the region in positive LD with the at-risk haplotype reconstructed in this study. The patterns of LD were shown to be two block like, the first block represented by 315C>T (covering rs2268583 at intron 1 to rs211037 at exon 5), and the second block represented by 1128+99C>A (covering rs2284780 at intron 7 to 1128+99C>A at intron 8). Since we found no association between each representative SNP and METH use disorder in either allelic or genotypic frequencies, the possibility arises that susceptibility variant can be located outside of these block-like regions. The second block includes the splicing regulatory elements surrounding the spliced exon, which bind to the polypyrimidine tract binding protein, the splicing regulator.[28–30] Actually, we screened this regulatory region thoroughly through direct sequencing of the 96 samples, however, could not find any variant in these elements. Other splicing regulatory elements that bind to another splicing regulator Nova-1 were located in intron 8, about 3.5 kb downstream of 1128+99C>A.[31,32] If the second block does not cover the latter splicing regulatory elements, these regions can be a susceptible candidate. Recently, a significant association was reported between rs4480617 at the 5′-UTR of GABRG2 and METH use disorder in females.[10] Therefore, this SNP or other variants in the promoter region also can be another candidate. Given that the sample size of 96 used to identify SNPs in this study provides more than 80% power to detect SNPs with about 1% minor allele frequency,[33] we are almost unlikely to overlook common nonsynonymous SNPs predisposed to METH use disorder.

As has been widely discussed, a spurious association can arise because of confounding such as population stratification and clinical heterogeneity, given the problems of reliability due to no use of structured interviews. However, our data are partly in agreement with a recent report[10] that found the significant association between GABRG2 and METH use disorder in females. This provides further corroboration that our haplotypic association with METH uses disorder is not spurious, although potential sources of bias such as ascertainment bias still remain possible. For example, subjects suffering from not only METH use disorder but also METH-induced psychosis are more likely to seek medical care and thus to be ascertained. Such 'spurious comorbidity'[34] of psychosis may account for the apparent association in this study. In the present study, we did not stratify the METH use disorder sample according to the comorbidity of METH-induced psychosis because the sample size was too small for reliable analysis. Although the precise prevalence of the comorbid METH-induced psychosis remains unknown, the data in the late 1940s and early 1950s in Japan indicating that about 10% of METH users had METH-induced psychosis[35] would suggest that comorbid METH-induced psychosis is over-represented in our clinically ascertained sample with METH use disorder.

As no association exists between GABRG2 and schizophrenia in our sample, association between GABRG2 and METH use disorder would not likely be attributable to spurious comorbid METH induced-psychosis, which may share the pathophysiology of susceptibility with schizophrenia, the so-called sensitization phenomena.[35] On the contrary, the comorbid polysubstance-related disorder over-represented in our sample with METH use disorder can account for the apparent association in this study. Indeed, previous findings suggesting nonspecific substance dependence vulnerability[5] supported the existence of such a 'misattributed' association in our study. In addition to concurrent comorbidity, we also cannot deny the possibility of spurious comorbid bias caused by the past comorbid diseases because of not examining the past history of any mental diseases systematically. METH use subjects in our study included a large number of patients who experienced first psychotic symptoms after METH use for a relatively short duration and participants in the special program designed for drug use disorder, in which they could not participate if they suffered from other psychiatric problems. The low levels of comorbidity in METH use subjects may reflect such biased ascertainment.

There is indeed a neuroscientific framework to link GABRG2 and METH use disorder. First, several lines of investigation[7] implicate the mesolimbic dopamine system in psychostimulant-induced motor activity. Furthermore, it was shown in a pharmacological study[36] that a GABAergic system in PFC modulated the motor response to psychostimulants by inhibiting PFC pyramidal neurons. Second, a tentative association was found for a GABRG2 SNP and the frontally located event-related potential (ERP) complex N100/P200 after auditory stimuli.[37] Thus, the prefrontal activation difference may reflect the differential GABRG2 activities derived from variants of the gene. Accordingly, GABRG2 activities in PFC could affect the modulation of mesolimbic reward circuitries, which might be associated with vulnerability of METH use disorder.

Overall our results indicate that GABRG2 may play a role in the risk of METH use disorder development in this population. Analysis of the promoter region or the splicing regulatory elements in intron 8 in a future study would be a logical next step in searching for a susceptible variant of GABRG2 in METH use disorder. However, it remains uncertain whether the associated phenotype may reflect the vulnerability of METH-specific abuse or nonspecific substance abuse.

METHODS

Subjects

All patients in this study were unrelated and recruited from three medical institutes participating the Japanese Genetics Initiative for Drug Abuse (JGAIDA).[38] They were diagnosed according to DSM-IV criteria by the consensus of at least two experienced psychiatrists on the basis of unstructured interviews and review of the medical records prior to genotyping.

The number of the patients with METH uses disorder, comprised of 164 METH-dependent subjects, and 14 METH abuse subjects, and schizophrenia were 178 (144 males and 34 females) and 288 (140 males and 148 females), respectively. The ages of each patient group were 18–69 years old (mean±SD; 36.7±12.0) and 15–75 (39.6±14.0), respectively. No patient with schizophrenia had severe physical complications or other Axis-I disorders according to DSM-IV when enrolled in this study, because seven schizophrenic subjects with METH use disorder were excluded based on the criteria that restricted a comorbid diagnosis of any psychotic disorder other than METH-induced psychosis. Among the subjects with METH use disorder, 150 (124 males and 25 females) have a comorbid diagnosis of METH-induced psychosis, three of anorexia nervosa, one of obsessive-compulsive disorder, and one of major depressive disorder. Additionally, 119 subjects with METH use disorder have abuse or dependence on drugs other than METH. The past history of any mental illness was not examined. The ages of METH-induced psychotic subgroup were 19–69 years old (37.7±12.3). No patient with METH use disorder had any severe physical complications when enrolled in this study. The 288 unrelated healthy volunteers (152 males and 136 females), aged 19–65 years (33.6±13.0), were comprised of hospital staff members and medical students at Fujita Health University. All healthy controls were also psychiatrically screened based on unstructured interviews. After complete description of the study to each subject, written informed consent was obtained. This study was approved by the ethics committee of each JGAIDA institute.

SNP Identification

Genomic DNA was isolated from whole blood using PUREGNER (Gentra system, Minneapolis, MN 55447, USA). For denaturing high-performance liquid chromatography (DHPLC) analysis, we designed specific primer sets amplifying all GABRG2 exons and the flanking intronic splice sites, based on GenBank sequence (NM000816 and NT023133) (primer sequences are available on request).

Polymerase chain reaction (PCR) was performed in a 10-μl volume containing 10 ng sample DNA, 0.4 M of each primer, 200 μM each dNTP, 1 × PCR Gold Buffer, 1.5 mM $MgCl_2$ and 0.25 U of Amplitaq Gold™ (Applied Biosystems Japan Ltd, Tokyo, Japan), using GeneAmp™ PCR system 9700 (Applied Biosystems Japan Ltd). PCR cycling conditions consisted of an initial denaturation step at 95°C for 9 min, followed by 45 cycles of 95°C for 15 s, 60°C for 20 s, 72°C for 30 s, and ending with a final extension step at 72°C for 7 min.

To screen for nucleotide variants, the obtained PCR products from all screened samples were analyzed by DHPLC with the WAVE™ system (Transgenomics Japan Ltd, Tokyo, Japan). The PCR products showing variant chromatograms were amplified again and then sequenced with an ABI PRISM™ 3100 Genetic Analyzer (Applied Biosystems Japan Ltd). Furthermore, to screen for any kinds of nucleotide variants in the splicing regulatory elements surrounding the spliced exon, we performed direct sequencing of the 96 controls. The conditions for DHPLC analysis and direct sequencing were reported previously.[39]

SNP Genotyping

To confirm the sequencing result and to genotype the variants in additional samples, the DHPLC analysis using the primer extension methods were developed for genotyping 588T>C by modifying the method of Hoogendoorn *et al*,[40] as reported previously.[39] All the remaining SNPs examined were genotyped using PCR-restriction fragment length polymorphism (PCR-RFLP) methods. Of four RFLP sites selected, the *Bam*HI restriction site in the eighth exon was genotyped as described by Loh *et al*,[12] while for the rest of the three SNPs, PCR-RFLP methods were developed (detailed information on experimental procedures is available upon request).

Statistical Analysis

Tests for Hardy–Weinberg equilibrium, the calculation of LD measures such as D′, Δ_2 and *P*-value and the estimation of haplotypic frequencies were carried out using Arlequin software 2.0.[41] The haplotypic frequencies between each patient group and controls were also compared using Arlequin software 2.0. The genotypic and allelic frequencies among each patient group and control group were compared with an exact test, using SPSS (version 10). A two-tailed level of 5% was chosen for the type I error rate. We have not corrected for multiple testing so as to avoid false negative findings.

Following Ohashi and Tokunaga,[40] we estimated the power of association analysis for our sample size of 178 subjects with METH use disorder, 288 schizophrenics and 288 controls under multiplicative model of inheritance, assuming a population susceptibility allele frequency of 0.30 at 315C>T and 0.48 at 1128+99C>A, the value in our screened samples. Setting the type I error rate at 5% and Genotype relative risk at more than 1.4 and 1.5, we obtained more than 80% power for direct association analysis of METH use disorder and schizophrenia, respectively.

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DUALITY OF INTEREST
None declared.

ABBREVIATIONS

METH	*methamphetamine*
GABA	*γ-aminobutyric acid*
GABRG2	*The human $GABA_A$ receptor gamma 2 subunit gene*
GAD	*glutamic acid decarboxylase*
PFC	*prefrontal cortex*
LD	*linkage disequilibrium*
DHPLC	*denaturing high-performance liquid chromatography*
PCR-RFLP	*polymerase chain reaction-restriction fragment length polymorphism*

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ORIGINAL ARTICLE

Linkage disequilibrium and association with methamphetamine dependence/psychosis of μ-opioid receptor gene polymorphisms

S Ide[1,2,3], H Kobayashi[1,4], H Ujike[5,17], N Ozaki[6,17], Y Sekine[7,17], T Inada[8,17], M Harano[9,17], T Komiyama[10,17], M Yamada[11,17], M Iyo[12,17], N Iwata[13,17], K Tanaka[1], H Shen[1], K Iwahashi[14], M Itokawa[15], M Minami[16], M Satoh[3], K Ikeda[1] and I Sora[1,4,17]

[1]Division of Psychobiology, Tokyo Institute of Psychiatry, Tokyo, Japan; [2]Laboratory of Neuropharmacology, Department of Pharmaceutical Sciences, Hiroshima International University, Kure, Japan; [3]Department of Molecular Pharmacology, Faculty of Pharmaceutical Sciences, Kyoto University, Kyoto, Japan; [4]Department of Psychobiology, Tohoku University Graduate School of Medicine, Sendai, Japan; [5]Department of Neuropsychiatry, Okayama University Graduate School of Medicine, Dentistry and Pharmaceutical Sciences, Okayama, Japan; [6]Department of Psychiatry, Nagoya University Graduate School of Medicine, Nagoya, Japan; [7]Department of Psychiatry and Neurology, Hamamatsu University School of Medicine, Hamamatsu, Japan; [8]Department of Psychiatry, Teikyo University, School of Medicine, Ichihara Hospital, Chiba, Japan; [9]Department of Neuropsychiatry, Kurume University School of Medicine, Kurume, Japan; [10]Division of Psychiatry, National Center Hospital for Mental, Nervous and Muscular Disorders, National Center of Neurology and Psychiatry, Tokyo, Japan; [11]Department of Psychogeriatrics, National Institute of Mental Health, National Center of Neurology and Psychiatry, Tokyo, Japan; [12]Department of Psychiatry, Graduate School of Medicine, Chiba University, Chiba, Japan; [13]Department of Psychiatry, Fujita Health University School of Medicine, Aichi, Japan; [14]Health Administration Center, Azabu University, Kanagawa, Japan; [15]Schizophrenia Research Project, Tokyo Institute of Psychiatry, Tokyo, Japan; [16]Department of Pharmacology, Hokkaido University Graduate School of Pharmaceutical Sciences, Sapporo, Japan and [17]Japanese Genetics Initiative for Drug Abuse (JGIDA), Japan

Correspondence:
Dr I Sora, Department of Psychobiology, Tohoku University Graduate School of Medicine, 1-1 Seiryo-machi, Aoba-ku, Sendai 980-8574, Japan.
E-mail: isora@mail.tains.tohoku.ac.jp

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Several studies indicate that the μ-opioid receptor plays a role in addiction not only to opiate drugs but also to alcohol and non-opiate addictive drugs. Our studies aim to reveal the associations between gene polymorphisms and methamphetamine (MAP) dependence/psychosis. We newly identified several polymorphisms and four substantial linkage disequilibrium (LD) blocks in the μ-opioid receptor (*OPRM1*) gene. We found significant differences in both genotype and allele frequencies of the single-nucleotide polymorphism (SNP) IVS2+G691C between control ($n=232$) and MAP-dependent/psychotic patients ($n=128$). There was also a significant association between IVS2+G691C and patients with transient psychosis. These results suggest that the *OPRM1* gene variations may be a factor in development and prognosis of MAP psychosis.
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Keywords: single-nucleotide polymorphism; human μ-opioid receptor; methamphetamine; dependence; psychosis

Introduction

Several studies indicate that the μ-opioid receptor plays a role in addiction not only to opiate drugs but also to non-opiate drugs of abuse.[1–5] Positive or negative regulation of the expression and/or function of the μ-opioid receptor may be involved in the mechanisms of drug dependence on both opiate and non-opiate drugs of abuse.[4,6,7] Several single-nucleotide polymorphisms (SNPs) that cause amino-acid substitutions (non-synonymous), synonymous SNPs and SNPs in the non-coding regions and introns in the μ-opioid receptor (*OPRM1*) gene have been reported in African-American and Caucasian populations.[7–9] Various studies of the association between frequencies of polymorphisms in the *OPRM1* gene and drug dependence on opioids, alcohol or other substances in African-American, Caucasian, Hispanic, Han Chinese and Swedish populations have been reported.[10–18] One of these SNPs, A118G, which alters the receptor function,[10] was associated with risk for drug abuse,[14,15] although contradictory data were also reported.[12,13,18]

Psychostimulants including methamphetamine (MAP) exert their reinforcing effects by modulating monoaminergic transmission, of which dopamine is supposed to be the most crucial.[19] The increase in dopamine release through dopamine transporters owing to MAP is primarily responsible for the induction of its reinforcing and psychogenic effects. Opioid receptor agonists are known to affect dopamine neurotransmission[20] and to attenuate MAP-induced enhance-

ment of dopamine neurotransmission.[21,22] Naloxone, an opioid receptor antagonist, potentiated the long-lasting dopamine depletion produced by MAP.[23] Furthermore, μ-opioid receptor-knockout mice display reduced reward from psychostimulants.[24] Other recent studies on animals also indicate complex interactions between the dopamine and opioid systems.[25–29] Therefore, it is possible that variations in the opioid receptor function could produce individual differences in the vulnerability to MAP dependence and/or psychosis. In a previous study, we found a significant association between the SNP A118G and the latency of MAP psychosis, although we did not find any significant association between the vulnerability to MAP dependence/psychosis and polymorphisms in exon 1 and its flanking regions of the *OPRM1* gene in Japanese subjects.[30] Recently, we identified the 3′-end of the major *OPRM1* transcript, MOR-1 mRNA.[31] In the present study, we extended the association studies to the downstream regions beyond the polyadenylation signal in the *OPRM1* gene to identify linkage disequilibrium (LD) blocks, and examined associations between polymorphisms in the *OPRM1* gene and MAP dependence and/or psychosis in Japanese.

Materials and methods

Subjects

One-hundred twenty-eight unrelated patients with MAP dependence/psychosis (99 males and 29 females, 35.9±1.0 years of age) meeting ICD-10-DCR criteria (F15.2 and F15.5) were used as case subjects; they were outpatients or inpatients of psychiatric hospitals. The patients had intravenously injected ($n=95$) or inhaled ($n=33$) MAP. All patients were diagnosed by two trained psychiatrists, and diagnoses were carried out on the basis of both interviews and all available information included in the hospital records. Patients were excluded if they had a clinical diagnosis of schizophrenia, another psychotic disorder or an organic mental syndrome. The 232 control subjects were mostly medical staff members who had neither personal nor familial history of drug dependence or psychotic disorders, as verified by a clinical interview. All subjects were Japanese, born and living in the northern Kyushu, Setouchi, Chukyo, Tokai and Kanto regions. This study was approved by the ethical 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 for this research.

Control samples were used to find *OPRM1* gene polymorphisms in the Japanese population. We first analyzed 44 controls to find polymorphisms and then employed 188 additional samples for intron 2 (total 232), 135 for intron 3 (total 179), and 53 (total 97) and 135 (total 179) for the 3′untranslated region (UTR), respectively, to estimate allelic frequencies.

For the association study, we used 179 controls (139 males and 40 females, 34.6±1.5 years of age) for the IVS3+6113, IVS3+8761, IVS3+G5953A and IVS3+A6151G polymorphic sites, 213 controls (166 males and 47 females, 34.8±1.6 years of age) for the A118G polymorphic site (in our previous study[30]) and 232 controls (181 males and 51 females, 35.2±1.8 years of age) for the IVS2+G691C polymorphic site. Gender, age and geographical distribution were matched without any known bias from a larger pool of control subjects.

Patient subgroups

We divided patients into two or three categories. The clinical characteristics of the subgroups were as follows:

(a) *Latency of psychosis from the first MAP intake*: The course of MAP psychosis varied among patients, and some patients showed psychosis sooner after the first MAP intake, as reported previously.[32,33] The median latency was 3 years. Therefore, patients were divided into two categories based on the latency of the psychotic state after the first MAP intake: less than 3 years ($n=54$, average=0.83 years) or more than 3 years ($n=53$, average=9.98 years).

(b) *Duration of psychosis after the last MAP intake*: Some patients showed continued psychotic symptoms despite MAP abstinence, as reported previously.[32,33] Liability for the duration of psychosis may be determined, at least partly, by genetic variation. We previously reported that genetic variation in the dopamine transporter affects the prognosis of the psychotic state.[32,33] Therefore, patients were divided into two categories: transient and prolonged psychosis after MAP abstinence. Patients with transient psychosis showed a reduction of psychotic symptoms within 1 month after the discontinuance of MAP consumption and the beginning of neuroleptic treatment, and prolonged psychosis continued for more than 1 month even after the discontinuance of MAP consumption and the beginning of neuroleptic treatment. In this study, 72 patients had the transient type and 43 had the prolonged type of MAP psychosis.

(c) *Spontaneous relapse*: It has been well documented that once MAP psychosis has developed, patients in the remission phase are liable to spontaneous relapse without MAP reconsumption.[32,33] It is postulated that a sensitization phenomenon induced by repeated consumption of MAP develops in the brains of MAP psychosis patients and is the neural basis for enhanced susceptibility to relapse.[34] Therefore, the patients were divided into two groups according to the presence ($n=86$) or absence ($n=42$) of spontaneous relapse.

(d) *Multiple-drug abuse*: We divided MAP-dependent/psychotic patients into three groups based on drug use, MAP only ($n=36$), MAP plus easily accessible legal substances (e.g., alcohol, inhalants, hypnotics; $n=49$) or MAP plus illegal substances that are relatively difficult to access in Japan (e.g., cocaine, heroin; $n=43$). Note that MAP is easier to obtain in Japan than other illegal substances (e.g., cocaine, cannabis, heroin). The total number of patients in each category was not always 128 because not all data were available for several patients.

Genotyping
Genomic DNAs were extracted from peripheral blood samples by using the standard phenol extraction protocol. Northern blot analysis has revealed that the single predominant transcript of the *OPRM1* gene is the approximately 14 kb long MOR-1 mRNA in the human brain.[35] We analyzed all exons of the MOR-1 mRNA and 5′ and 3′ intronic regions of each exon. We also analyzed the parts of the intron-containing nucleotide repeats, which were found in the database for genomic contig sequence (Genbank Accession No. NT-025741). We independently screened the amino-acid coding regions of the *OPRM1* gene for sequence variations: exon 1 (290 bp) (in a previous study[30]), exons 2–3 (353 and 521 bp), exon 4 (39 bp) and a part (from the stop codon to 2951 bp downstream from the stop codon) of the 3′UTR in exon 4 (Figure 1). The neighboring intron sequences of each exon, intron 2 (773 bp) and some parts of intron 3 were also screened for sequence variations. In order to screen for all possible polymorphisms in the *OPRM1* gene, we first analyzed all regions of all PCR products of genomic DNA from 44 control subjects by using an automated DNA sequencer. Then, the regions that included frequent polymorphisms (allelic frequency >5%) and all exons were analyzed by an automated DNA sequencer using the genomic DNAs of the remaining control subjects. The rest of the 3′UTR of the exon 4 (10 682 bp) and the 197 bp 3′-flanking region (total 10 879 bp) of the gene were similarly analyzed with DNA samples of 12 control subjects and 36 primers. Primers were designed on the basis of the reference genomic contig sequence in the National Center of Biotechnology Information (Genbank Accession No. NT-025741). The LD blocks of the polymorphisms were estimated using HaploBlockFinder Version 0.7 (http://cgi.uc.edu/cgi-bin/kzhang/haploBlockFinder.cgi). D' and r^2 were calculated by using the appropriate formula in the Excel program.[36]

Statistical analysis
The χ^2 test was used for statistical analysis. The statistical significance level was set at $\alpha = 0.05$, and Bonferroni corrections were conducted on association analyses. The Hardy–Weinberg (HW) equilibrium was determined by using the χ^2 test. In the analysis of LD and estimation of haplotype frequencies, genotypic data from 179 control subjects were analyzed by using the Arlequin program available from http://anthro.unige.ch/arlequin.[37] The effect size and power calculations were performed by using the G*Power program (set α value as 0.05) available from http://www.psycho.uni-duesseldorf.de/aap/projects/gpower/.[38]

Results

Analysis of OPRM1 *gene sequence variation*
We analyzed exons 2–3 of the MOR-1 mRNA, parts of the introns and parts of exon 4 of the *OPRM1* gene using genomic DNA samples of Japanese control subjects. We found 19 SNPs (Table 1(1)), one dinucleotide polymorphism (DNP), IVS3+6113 $(GT)_{11-15}$ and one polynucleotide polymorphism (PNP), a 32-base pair repeat IVS3+8761 $(GAC\ ATA\ TAT\ CAT\ AAT\ ATA\ TAT\ TAT\ CAT\ ATT\ AT)_{2-17}$ (Table 1(2)). None of the polymorphisms deviated from the HW expectations. There was no significant difference in allelic frequencies of polymorphisms between subjects from the Setouchi and Kanto regions, where the majority of our present subjects were born and are living ($P > 0.4$; χ^2 tests). The allelic frequency of SNP IVS2+G691C was remarkably higher in Japanese control subjects (81.5%) than in African-American or Caucasian populations (42.5–53.3%).[9,11] We did not find any SNP in the amino-acid coding regions of the *OPRM1* gene in Japanese subjects, except the SNP A118G reported previously.[30]

OPRM1 *genotypes and LD*
LD was quantified by using Lewontin's standardized coefficient D' (Figure 1). We identified a block of LD at the *OPRM1* locus that extended from intron 3 to the 3′UTR. The SNPs (IVS3-G8804A, TAA+G886A, T1371C, G1670A, C2007T, A2109G, G2287A and G2395C), in intron 3 and the 3′UTR, which spanned an 11-kbp sequence, were observed to be in a complete LD relationship (all possible $D' = 1.000$). The remaining SNPs within the region, except TAA+A2109G (TAA+1260, 1709, 2274), showed a high LD, suggesting that these SNPs were also in the LD block. To determine the extent of the LD block in the 3′ downstream region, we analyzed sequence variations up to 14 kbp downstream from the stop codon (TAA) with DNA samples of 12 control subjects. We found 42 polymorphisms in this region, and of them, 27 were in absolute LD (all possible $D' = 1.000$ and $r^2 = 1.000$, data not shown).

One representative SNP (TAA+A2109G) was selected from the polymorphisms in absolute LD. Seven SNPs in the rest of the *OPRM1* gene (the 5′-flanking region to the SNP IVS3-G8804A) showed over 5% allelic frequency. Thus, we selected these eight SNPs to calculate D' values. LD analyses showed that LD relationships with significant D' values were observed for all possible combinations of SNPs, except for IVS3+G5953A and IVS3+G8497T (Table 2). LD relationships with significant r^2 values were also observed for the combinations of TAA+A2109G with IVS3+A6151G or IVS3+A8449G. The matrix of D' values revealed a contiguous block of high values ($D' > 0.97$) for four sequential markers, IVS3+A6151G to TAA+A2109G. Two regions, from A118G to IVS3+G5807A and from IVS2+G691C to IVS3+G5953A, were also in LD blocks with high D' values ($D' > 0.7$). Interestingly, in spite of the diversity of the number of repeats, there were also significant LDs ($P < 0.05$ by the Arlequin program) between the DNP and PNP in intron 3 and the eight SNPs (Figure 1), as in the case of the DNP in intron 1.[26] These results suggest at least three recombination positions between A118G and IVS2+G691, IVS3+G5807A and IVS3+G5953A, and IVS3+G5953A and IVS3+A6151G. The following LD blocks were found in the *OPRM1* gene by haplotype analysis using the HaploBlockFinder Version 0.7 program: (A) the region containing SNP A118G (around exon 1), (B) the region containing

Table 1 Polymorphisms found in the *OPRM1* gene of control subjects: (1) list of SNPs and (2) DNP and PNP

Position	*SNP name*	*Allelic frequency (%)*	*Sample size*	*Reported allelic frequency (%)*	*rs number*	*References*
(1) List of SNPs						
Exon 1	A118G	45.3	213	7.5–25.8	rs 1799971	10–12,15,26
Intron 2	IVS2+G31A	2.6	232	4.2	rs 9479757	15
	IVS2+G518A	<1.5	232			
	IVS2+G691C	81.9	232	42.5–53.3	rs 2075572	9,11
Intron 3	IVS3+G5807A	44.4	179		rs 3798683	
	IVS3+G5953A	13.1	179		rs 599548	
	IVS3+A6151G	92.5	179		rs 598682	
	IVS3+A8449G	9.2	179		rs 9384179	
	IVS3+C8497T	31.3	179		rs 9371774	
	IVS3−G8804A	9.5	179		rs 609148	
3′UTR	TAA+G886A	10.8	97			
	TAA+T1306C	<1.5	97			
	TAA+T1371C	10.8	97			
	TAA+G1670A	11.4	44			
	TAA+G1709A	5.7	44			
	TAA+C2007T	11.4	44			
	TAA+A2109G	8.4	179			
	TAA+A2274G	11.7	179			
	TAA+G2287A	90.8	179			
	TAA+G2395C	8.4	179			

(2) DNP and PNP	*Number of repeats*	*11*	*12*	*13*	*14*	*15*
IVS3+6113 (GT)*n*	Control (2*n*=358) (%)	35 (9.8)	1 (0.3)	284 (79.3)	33 (9.2)	5 (1.4)

	Number of repeats	*2*	*—*	*8*	*9*	*10*	*11*	*12*	*13*	*14*	*15*	*16*	*17*
IVS3+8761 (32 bp)*n*	Control (2*n*=358) (%)	34 (9.5)	—	1 (0.3)	12 (3.4)	100 (27.9)	33 (9.2)	118 (33.0)	18 (5.0)	9 (2.5)	6 (1.7)	25 (7.0)	2 (0.6)

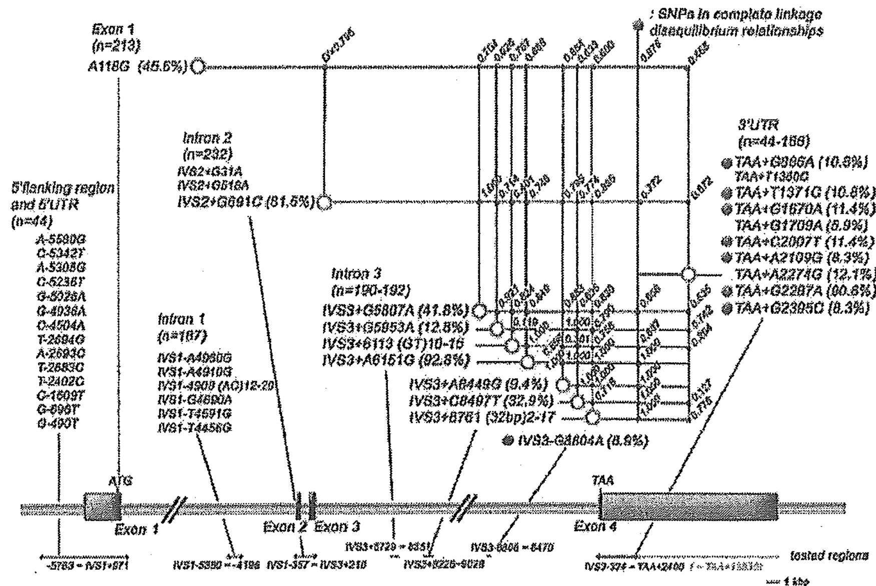

Figure 1 Allelic frequencies and LD relationships of polymorphisms in the *OPRM1* genes of Japanese control subjects. Tested regions (the 5′-flanking region, exon 1 and part of intron 1 were examined in a previous study[30]) are indicated under the scheme of the *OPRM1* gene, and allelic frequencies are shown in the brackets after each polymorphism. The numbers near the small black dots at the crossing of lines represent D' values for each combination of polymorphisms. Polymorphisms in the region from TAA+2400 to TAA+13 830 (gray bar in tested regions) are not shown because of the small number of subjects ($n=12$).

Table 2 LD among SNPs in *OPRM1* gene of control subjects

Locus	A118G	IVS2+ G691C	IVS3+ G5807A	IVS3+ G5953A	IVS3+ A6151G	IVS3+ A8449G	IVS3+ C8497T	TAA+ A2109G	TAA+ A2274G	TAA+ G2287A
	D' (upper) / r^2 (lower)									
A118G		0.795	0.704	0.626	0.866	0.884	0.633	0.875	0.458	0.890
IVS2+G691C	0.125		1.000	0.714	0.746	0.795	0.775	0.772	0.672	0.795
IVS3+G5807A	0.453	0.134		0.921	0.849	0.883	0.895	0.868	0.535	0.883
IVS3+G5953A	0.052	0.341	0.102		0.023	0.009	1.000	0.034	0.742	0.009
IVS3+A6151G	0.054	0.201	0.047	0.000		1.000	1.000	1.000	1.000	1.000
IVS3+A8449G	0.069	0.284	0.063	0.000	0.001		1.000	1.000	1.000	0.967
IVS3+C8497T	0.159	0.062	0.291	0.069	0.037	0.046		1.000	0.763	1.000
TAA+A2109G	0.061	0.241	0.055	0.001	0.892	0.901	0.042		1.000	1.000
TAA+A2274G	0.024	0.266	0.030	0.484	0.011	0.014	0.035	0.014		1.000
TAA+G2287A	0.070	0.284	0.063	0.000	0.800	0.934	0.046	0.989	0.014	

Light gray shading indicates a significant LD with a high D' (>0.7) and dark gray shading indicates that both D' and r^2 values are high.

IVS2+C691G and IVS3+G5807A (at least 6.4 kb from intron 2 to about 5.9 kb 3′ downstream from the exon 3–intron 3 junction), (C) the region containing IVS3+G5953A (around 6 kb 3′ downstream from the exon 3–intron 3 junction) and (D) the region containing other SNPs (at least 23.4 from 6.1 kb 3′ downstream from the exon 3–intron 3 junction to the 3′-end of exon 4).

Relationship between OPRM1 *gene polymorphisms and MAP dependence/psychosis*

We selected four representative SNPs (A118G, IVS2+G691C, IVS3+G5953A and IVS3+A6151G) from the estimated LD blocks by using HaploBlockFinder to study the association between polymorphisms in the *OPRM1* gene and MAP dependence/psychosis. We found significant differences in both genotype and allele frequency at IVS2+G691C (χ^2 test, $P=0.012$ and 0.011, respectively) between the controls and all patients with MAP dependence/psychosis (Table 3). These significances were also shown after the Bonferroni correction (corrected significance level was $\alpha=0.0125$). The calculated statistical powers for the SNPs A118G, IVS2+G691C, IVS3+G5953A and IVS3+A6151G were 0.67, 1.00, 0.41 and 0.93, respectively. Although we further estimated the haplotypes and calculated D' values of these

four SNPs, there was no significant difference ($P=0.30$) in haplotypes between the control and MAP-dependent/psychotic subjects (Table 4).

We also studied the association between polymorphisms in the *OPRM1* gene and the clinical parameters (latency of psychosis, psychosis prognosis, spontaneous relapse and multiple-substance abuse). The significant associations are presented in Table 5. Based on the latency of psychosis, three SNPs (A118G, IVS2+G691C and IVS3+A6151G) showed significant differences in genotype frequencies (χ^2 test, $P=0.040$, 0.0085 and 0.027, respectively) between the control and MAP-dependent subjects whose psychosis appeared less than 3 years from the first MAP intake. There was also a significant difference ($P=0.039$) in the SNP IVS2+G691C between the control and MAP subjects whose psychosis appeared more than 3 years after the first MAP intake, but this significance level was lower than that for the short latency group. In the prognosis of psychosis subgroups, only one SNP, IVS2+G691C, showed a significant difference in genotype frequency ($P=0.0034$) between the control and MAP-dependent subjects whose psychosis continued for less than 1 month after the last MAP intake. The association between SNP IVS2+G691C and the transient course of MAP psychosis was still statistically significant after the Bonferroni correction (corrected significance level was $\alpha=0.00625$). Similarly, in the spontaneous relapse subgroups, only the SNP IVS2+G691C showed a significant difference in genotype frequency ($P=0.014$) between the control and MAP subjects with spontaneous relapse. In the multiple-drug abuse subgroups, SNP IVS2+G691C again showed a significant difference in genotype frequency ($P=0.0059$) between the control and MAP-dependent/psychotic subjects who did not use any other drug. Most analyses of subcategories showed enough statistical power to detect a small size effect, although some showed weaker power (A118G for MAP-dependent/psychotic subjects who did not use any other drug, (0.31) IVS3+G5953A for transient prognosis of psychosis (0.29) or for no spontaneous relapse, (0.13) and IVS3+A6151G for later appearance of psychosis (0.16)).

Discussion

Recently, LD mapping has proved to be a powerful tool for identifying genes underlying complex clinical traits.[39,40] The identification of at least four LD blocks in the *OPRM1* gene in the present study may be useful in future association studies of polymorphisms in the *OPRM1* gene with other clinical traits. The incidence of recombination may be high in the *OPRM1* gene relative to other genes owing to its telomeric position on chromosome 6 (6q24–q25). However, the present study indicated that a high degree of LD still exists within the *OPRM1* gene. This preservation of LD was

Table 3 Allelic and genotypic frequencies of SNPs in control and MAP-dependent/psychotic subjects

Locus	*Control subjects*			*MAP-dependent/psychotic subjects*				
	Genotypic data		*Allelic data*	*Genotypic data*			*Allelic data*	
	Number	*Allelic frequency (%)*	*Number*	*Number*	*Allelic frequency (%)*	*P-value*	*Number*	*P-value*
	A 67 (0.31)		A 233 (0.55)	A 49 (0.38)			A 152 (0.59)	
A118G	A/G 99 (0.46)	45.3	G 193 (0.45)	A/G 54 (0.43)	40.6		G 104 (0.41)	
	G 47 (0.22)			G 25 (0.19)		$P=0.43$		$P=0.23$
	Total 213		Total 426	Total 128			Total 256	
	G 6 (0.03)		G 84 (0.18)	G 12 (0.09)			G 67 (0.26)	
IVS2+G691C	G/C 72 (0.31)	81.9	C 380 (0.82)	G/C 43 (0.33)	73.8		C 189 (0.74)	
	C 154 (0.66)			C 73 (0.57)		$P=0.012$		$P=0.011$
	Total 232		464	Total 128			Total 256	
	G 135 (0.75)		G 311 (0.87)	G 96 (0.75)			G 220 (0.86)	
IVS3+G5953A	A/G 41 (0.23)	13.1	A 47 (0.13)	A/G 28 (0.22)	14.1		A 36 (0.14)	
	A 3 (0.02)			A 4 (0.03)		$P=0.70$		$P=0.74$
	Total 179		Total 358	Total 128			Total 256	
	A 2 (0.01)		A 27 (0.08)	A 1 (0.01)			A 28 (0.11)	
IVS3+A6151G	A/G 23 (0.13)	92.5	G 331 (0.92)	A/G 26 (0.20)	89.1		G 228 (0.89)	
	G 154 (0.86)			G 101 (0.79)		$P=0.21$		$P=0.15$
	Total 179		Total 358	Total 128			Total 256	

Table 4 Haplotype analysis in *OPRM1* gene of control and MAP-dependent/psychotic subjects

	Control Frequency (%)	MAP dependence/psychosis Frequency (%)	Haplotype			
			A118G	IVS2+G691C	IVS3+G5953A	IVS3+A6151G
1	0.2	0.0	A	C	A	A
2	2.4	0.5	A	C	A	G
3	1.4	0.0	A	C	G	A
4	32.9	33.6	A	C	G	G
5	0.5	0.0	A	G	A	A
6	8.0	11.5	A	G	A	G
7	5.1	10.9	A	G	G	A
8	2.9	2.8	A	G	G	G
9	0.6	1.5	G	C	A	G
10	44.1	38.2	G	C	G	G
11	1.5	0.5	G	G	A	G
12	0.4	0.0	G	G	G	A
13	0.0	0.4	G	G	G	G

exemplified in the 3′ region of the gene. We found absolute LD among polymorphisms in the region spanning nearly 31 kb from intron 3 to beyond the end of the 3′UTR (TAA+13.6 kb). This indicates that recombination has been remarkably rare within the block. This long and absolute LD block suggests that a set of many polymorphisms in the block may affect the expression level of the *OPRM1* gene.

We found a significant association between the SNP IVS2+G691C in intron 2 and MAP dependence/psychosis even after the Bonferroni correction in the present study. This result provides pharmacogenetic evidence for cross-talk between the opioid and dopamine systems, as has been suggested by other studies.[20,27–29,41,42] On the other hand, the SNP A118G was not significantly associated with MAP dependence/psychosis, as shown in our previous report.[30] These results, taken together with the fact that the SNP A118G was found only in the coding regions of the *OPRM1* gene in Japanese subjects, could suggest that polymorphisms in the non-coding regions rather than in the coding regions of the *OPRM1* gene affect MAP dependence/psychosis.

Use of MAP induces a strong psychological dependence, and repeated use frequently results in psychotic states, symptoms of which are similar to those seen in schizophrenia of the paranoid type.[32,34] Interestingly, the SNP IVS2+G691C was significantly associated with the transient course of MAP psychosis even after the Bonferroni correction. We consider that the SNPs A118G, IVS2+G691C and IVS3+A6151G tend to be associated with the short latency of MAP psychosis. Similarly, the SNP IVS2+G691C tended to be associated with the spontaneous relapse of MAP psychosis. Although these differences were not significant after the Bonferroni correction, we think that these tendencies reflect the existence of an association between *OPRM1* polymorphisms and MAP psychosis. It has also been reported that the Bonferroni correction could be conservative for SNPs in LD.[43] Taken together, these significant differences and tendencies toward differences suggest that these SNPs might be related to the severity of MAP psychosis. In particular, the SNP IVS2+G691C might be related to a rapid induction of psychosis. Furthermore, the SNP IVS2+C691G had a tendency to be associated with single-drug abusers of MAP rather than multiple-drug abusers in the present study. This result is consistent with previous reports that the association was not observed in individuals with alcohol and drug dependence who were multiple-drug abusers.[9,11] There are reports that increased frequency of MAP use facilitated the appearance of MAP psychosis.[44,45] However, the information available on our subjects was not sufficient to confirm the relation between the frequency of MAP use and MAP psychosis. Although the statistical power for most analyses of subcategories of MAP psychosis was sufficient to detect an association, there is some possibility that a Type 1 error, the false rejection of the null hypothesis, exists in analyses of some subcategories with weaker powers. Further study with a larger number of subjects is required to determine the detailed association of these SNPs with the subcategories. Although it is unclear whether or not MAP dependence susceptibility has an association with the polymorphisms of the *OPRM1* gene found in our present analysis using MAP dependent/psychotic subjects, the present results of subcategory analyses suggest that the opioid system may be involved in vulnerability to MAP psychosis.

The mechanisms underlying the relationship between the SNP IVS2+G691C and vulnerability to MAP dependence/psychosis have not yet been clarified. It has been revealed that the sequence variation in the amino-acid non-coding region of the murine μ-opioid receptor (*Oprm1*) gene caused changes in the expression level of mRNA and the analgesic effect of morphine among different strains of mice.[46,47] Furthermore, scatter plot analysis for comparison of the *OPRM1/Oprm1* genes showed highly conserved regions between these species.[7,31] One of the possible mechanisms is that the SNP IVS2+G691C, and/or other polymorphisms in the LD block, might be located on the sites of DNA-

Table 5 Association between polymorphisms and clinical parameters of MAP psychosis

Locus		Control subjects		MAP dependent/psychotic								
				Group 1			Group 2			Group 3		
		Number	Frequency (%)	Number	Frequency (%)		Number	Frequency (%)		Number	Frequency (%)	
(1) Latency of psychosis				*< 3 years*			*≥ 3 years*					
A118G	A	67 (0.31)		25 (0.46)			20 (0.38)					
	A/G	99 (0.46)	45.3	24 (0.44)	31.5	$P=0.0404$	25 (0.47)	38.7	$P=0.4691$			
	G	47 (0.22)		5 (0.09)			8 (0.15)					
	Total	213		54			53					
IVS2+G691C	G	6 (0.03)		6 (0.11)			5 (0.09)					
	G/C	72 (0.31)	81.9	20 (0.37)	70.4	$P=0.0085$	19 (0.36)	72.6	$P=0.0394$			
	C	154 (0.66)		28 (0.52)			29 (0.55)					
	Total	232		54			53					
IVS3+A6151G	A	2 (0.01)		0 (0.00)			1 (0.019)					
	G/A	23 (0.13)	92.5	15 (0.28)	86.1	$P=0.0269$	7 (0.132)	91.5	$P=0.9060$			
	G	154 (0.86)		39 (0.72)			45 (0.849)					
	Total	179		54			53					
(2) Prognosis of psychosis					*Transient*			*Prolonged*				
IVS2+G691C	G	6 (0.03)		8 (0.11)			3 (0.07)					
	G/C	72 (0.31)	81.9	27 (0.38)	70.1	$P=0.0034$	13 (0.30)	77.9	$P=0.3303$			
	C	154 (0.66)		37 (0.51)			27 (0.63)					
	Total	232		72			43					
(3) Spontaneous relapse					*Non-existent*			*Existent*				
IVS2+G691C	G	6 (0.03)		6 (0.07)			5 (0.12)					
	G/C	72 (0.31)	81.9	30 (0.35)	75.6	$P=0.1250$	14 (0.33)	71.4	$P=0.0143$			
	C	154 (0.66)		50 (0.58)			23 (0.55)					
	Total	232		86			42		42			
(4) Poly-drug abuse					*None*			*Easily accessible drug*			*Drug difficult to obtain*	
IVS2+G691C	G	6 (0.03)		5 (0.14)			3 (0.06)			1 (0.02)		
	G/C	72 (0.31)	81.9	11 (0.31)	70.8	$P=0.0059$	15 (0.31)	78.6	$P=0.4401$	18 (0.42)	76.7	$P=0.3801$
	C	154 (0.66)		20 (0.56)			31 (0.63)			24 (0.56)		
	Total	232		36			49			43		

binding proteins and/or other modulators, and thus can change the expression level of the *OPRM1* gene. A comparison of expression levels of the *OPRM1* gene in individuals with the different forms of the variants may help to elucidate the roles of the variants.

Twenty-thousand MAP abusers in Japan have been arrested annually in the last 30 years. The number of MAP abusers in Japan has recently been stable, although the number was larger at the end of the Second World War.[45,48] In Japanese MAP abusers, single-drug abuse is the most common form, because MAP is relatively easy to obtain among illegal substances, whereas other illegal substances (e.g., cocaine, cannabis, heroin) are quite difficult to obtain. In Japan, the number of MAP abusers arrested was 100 times more than abusers of cocaine or cannabis. MAP abuse is not limited to specific Japanese populations. For example, high school students have recently started to use MAP casually to lose weight or just for fun.[45] Further, more than 80% of MAP abusers show MAP psychosis, and MAP may be one of the causes of the recent increase in crime. Thus, MAP abuse is a very serious and noticeable social problem in Japan. The present findings may lead to a better understanding of genetic vulnerability to MAP abuse and might provide suggestions to solve this problem.

The cross-talk between the opioid and dopamine systems underscores the role of the μ-opioid receptor in dependence on non-opiate drugs. The present findings suggest that the sequence variation of the *OPRM1* gene may be useful as a genetic marker to predict the development and prognosis of MAP psychosis.

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Duality of interest

None declared.

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No association was found between a functional SNP in ZDHHC8 and schizophrenia in a Japanese case–control population

Shinichi Saito[a,1], Masashi Ikeda[a,b,1], Nakao Iwata[b], Tatsuyo Suzuki[b], Tsuyoshi Kitajima[b], Yoshio Yamanouchi[b], Yoko Kinoshita[b], Nagahide Takahashi[a], Toshiya Inada[a,*], Norio Ozaki[a]

[a] *Department of Psychiatry and Psychobiology, Nagoya University Graduate School of Medicine, Nagoya 466-8550, Japan*
[b] *Department of Psychiatry, Fujita Health University School of Medicine, Toyoake, Aichi 470-1192, Japan*

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Abstract

ZDHHC8 is a new and attractive candidate for a schizophrenia-susceptibility factor. First, several lines of linkage studies showed that 22q11, on which ZDHHC8 is located, is a "hot" region. Second, fine linkage disequilibrium mapping revealed a significant association around ZDHHC8. Moreover, a very recent study reported that one single nucleotide polymorphism (SNP: rs175174) in ZDHHC8 might affect the splicing process, the ZDHHC8 knock-out mice showed the gender-specific phenotype, and the transmission disequilibrium test (TDT) using this SNP also showed significant association with human female schizophrenia. Thus, we attempted a replication study of this SNP using relatively large Japanese case–control samples (561 schizophrenics and 529 controls). No association was found between schizophrenia and controls even after dividing samples by gender. Because our sample size provided quite high power, ZDHHC8 may not play a major role in Japanese schizophrenia. And our results did not support the gender-specific effect of this SNP.
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Keywords: Chromosome 22q11; Gender difference; Candidate gene

The 22q11 region (OMIM: #600850 SCZD4) is associated with increased risk for schizophrenia [2]. Two independent meta-analyses of linkage studies showed the linkage around 22q11 [1,5], although one negative result was also reported [8]. This chromosome region contains at least three genes, COMT [12], PRODH2 and DGCR6 [7], implicated as susceptibility genes for schizophrenia.

Recently, ZDHHC8 was reported as a new and attractive candidate gene on 22q11 from the evidence of a genetic association study and animal study [6,9]. In the initial genetic association study, Liu et al. showed that three single nucleotide polymorphisms (SNPs) in ZDHHC8 were associated with schizophrenia. One of these SNPs (rs175174), which was located in intron 4 of ZDHHC8, showed the most highly significant *P* value [6]. This intronic SNP seemed to modify ZDHHC8 expression by causing imperfect splicing, intron retention and reduced enzyme activity. In addition, *Zdhhc8* knockout mice had a gender-dependent dimorphic deficit in prepulse inhibition similar to schizophrenia and reactivity to the psychomimetic *N*-methyl-D-aspartate (NMDA) receptor blocker dizocilpine. In the light of these findings, the transmission disequilibrium test (TDT) divided samples according to gender differences, revealing that human female schizophrenia was significantly associated with this SNP [9]. Thus, we here provide a replication study of rs175174 in ZDHHC8 using Japanese case–control samples.

A total of 561 patients with schizophrenia (259 female; mean age ± standard deviation (S.D.) 49.6 ± 16.4 years: 302

* Corresponding author. Tel.: +81 52 744 2284; fax: +81 52 744 2293.
E-mail address: inada@med.nagoya-u.ac.jp (T. Inada).
[1] These authors contributed equally to this work.

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male; 47.0 ± 14.9) and 529 controls (270 female; 39.7 ± 15.4 years: 259 male; 34.9 ± 12.4 years) were genotyped for association analysis of rs175174. Moreover, in additional linkage disequilibrium (LD) mapping around this SNP, 95 schizophrenic patients (50 female and 45 male) and 96 controls (44 female and 52 male), part of each sample used in association analysis, were genotyped for three SNPs. The general characterization of these subjects and a description of their psychiatric assessment according to identical criteria were published elsewhere [13]. After explaining the study to all subjects, written informed consent was obtained from each. This study was approved by the Ethics Committee of the Nagoya University Graduate School of Medicine and Fujita Health University.

Genomic DNA was extracted from peripheral blood of all subjects. For rapid genotyping of SNPs, rs175174 and additional three SNPs for LD mapping (rs175169, rs175175 and rs2292570), polymerase chain reaction (PCR)-restriction fragment length polymorphism (RFLP) assays were developed. The information of PCR primers is available on request. The PCR reactions of all SNPs were carried out in a 10 μl volume containing 10 ng genomic DNA, 0.4 M of each primer, 200 μM of dNTP, 1× PCR Gold Buffer, 1.5 mM $MgCl_2$ and 0.25 U of Amplitaq Gold™ (Applied Biosystems Japan Ltd., Tokyo, Japan), using the GeneAmp™ PCR system 9700 (Applied Biosystems Japan Ltd.). PCR cycling conditions consisted of an initial denaturation step at 95 °C for 9 min, followed by 45 cycles of 95 °C for 15 s, 56 °C for 20 s, 72 °C for 30 s, and ending with a final extension step at 72 °C for 7 min. PCR product was digested using appropriate restriction enzymes according to the manufacture's recommendation (New England Biolabs, England, UK) (Table 1). DNA fragments were resolved by electrophoresis in a 6% acrylamide gel stained with ethidium bromide.

Genotype deviation from the Hardy–Weinberg equilibrium (HWE) was evaluated by χ^2 test. Marker-trait association analysis was also evaluated by χ^2 test (SPSS 10.0J, SPSS Japan Inc., Japan). To evaluate pairwise LD matrices among SNPs (by D' and r^2), we used the software HAPLOVIEW version 2.05 (developed in Mark Daly's lab., URL; http://www.broad.mit.edu/personal/jcbarret/haploview/index.php). This software also defined "LD blocks" by reasonable criteria based on 95% confidential bounds on D' values [4]. Power calculation was performed using a statistical program prepared by Ohashi et al. [10]. The significance level for all statistical tests was 0.05.

In view of the gender differences in gene effects, we included analyses of samples divided according to the gender. Both in cases and controls, genotype frequencies of total, female and male samples were not significantly different from HWE.

In association analysis, we could not find associations of rs175174 with schizophrenia in either male or female (Table 2).

Next, to test whether rs175174 is representative for ZDHHC8 or not, we performed LD mapping using three additional SNPs around ZDHHC8 (Fig. 1). LD matrices between each pair of SNPs showed strong LD both in cases and controls (Table 1). Even after dividing samples according to the gender, all LD patterns showed the same trends (data not shown). These findings may suggest that the LD pattern of ZDHHC8 is a block-like pattern and that rs175174 is the "representative SNP" of this gene.

The power based on genotype relative risk (GRR) was calculated to evaluate the non-significant results due to type II error. When we set the GRR at 1.28, 1.42 and 1.40 in all, female and male samples, respectively (multiplicative model), our sample size provided powers of more than 80%.

We could not replicate an original positive association using TDT of ZDHHC8 with schizophrenia by the present case–control association analysis among Japanese. Nor could we replicate the gender-specific effect of the risk SNP. In this association analysis, our sample sizes provide enough power to deny the hypothesis. We also performed the fine LD mapping of Japanese samples and showed that the LD pattern of ZDHHC8 was the same block-like pattern as one of the samples from the United States and South Africa. The results provide evidence that not only rs175174 but also ZDHHC8 would not be a susceptibility factor for schizophrenia in either Japanese females or males. The discrepancy between Japanese and the samples from the United States and South Africa may derive from ethnic differences.

A couple of limitation should be addressed to discuss the present results. Initially, the mean age of controls is much younger than that of patients in the present study. This means that a number of young controls, although not more than five subjects given a lifetime morbidity risk of 0.8–1.0%, may go on to develop schizophrenia. This confounding factor might weaken the power of the present study. Another limitation

Table 1
SNPs in LD mapping and pairwise LD matrices

SNP ID	D'				Restriction enzyme
	rs175169	rs175174	rs175175	rs2292570	
rs175169		0.97 (0.78)	1.0 (0.29)	1.0 (0.67)	*Bsl*I
rs175174	0.97 (0.80)		1.0 (0.36)	1.0 (0.58)	*Bse*RI
rs175175	1.0 (0.26)	1.0 (0.31)		1.0 (0.21)	*Alw*NI
rs2292570	0.93 (0.76)	0.97 (0.70)	1.0 (0.23)		*Tsp*RI

Upper diagonal figures are D' (r^2) of controls and lower diagonal figures are D' (r^2) of schizophrenia.

Table 2
Association analysis of rs175174

Samples	Number	G/G	G/A	A/A	P value (genotype)	MAF[a]	P value (allele)
Total							
SCZ	561	238	245	78		0.357	
CON	529	205	259	65	0.213	0.368	0.618
Female							
SCZ	259	114	106	39		0.355	
CON	270	112	130	28	0.133	0.344	0.714
Male							
SCZ	302	124	139	39		0.359	
CON	259	93	129	37	0.457	0.392	0.260

SCZ: schizophrenia; CON: control.
[a] Minor allele frequency.

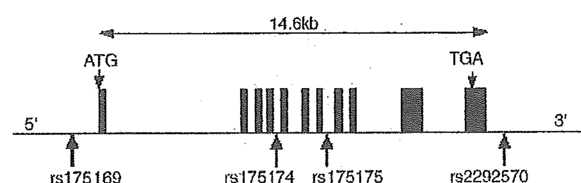

Fig. 1. Genomic structure of ZDHHC8 and SNPs used in association analysis and LD mapping. Vertical bars represent exons of ZDHHC8, and each number under arrows represents SNP ID.

which must be exercised is that the other candidates related to the neurodevelopmental and neuroprotective effect of ZDHHC8 would be in locus heterogeneity [11]. For example, ZDHHC8 encodes a putative transmembrane palmitoyltransferase modulating numerous classes of neuronal proteins including proteins important for neuronal development, neurotransmitter receptors such as NMDA [3]. Thus, the combined effect between ZDHHC8 and the other genes might be a stronger predisposing factor. Further genetic analysis including related candidate genes would definitely be required for a conclusive result.

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