Addiction Biology (June 2005) 10, 145–148

RESEARCH ARTICLE

Functional polymorphism of the NQO2 gene is associated with methamphetamine psychosis

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Abstract

Several lines of evidence suggest that genetic factors contribute to the vulnerability of drug abuse such as methamphetamine (MAP), and that dopamine-quinones produced by administration of MAP may be involved in the mechanism of MAP-related symptoms. The detoxification of quinones is catalyzed by a family of proteins designated as quinone oxidoreductases (NQOs). We analysed the polymorphisms of NQO1 and NQO2 genes to elucidate the association with genetic vulnerability to MAP abuse in Japan. The genotype and allele frequencies for the polymorphism (Pro187Ser) of the NQO1 gene did not differ between each subgroup of patients and controls. In contrast, the genotype frequency for the insertion/deletion (I/D) polymorphism in the promoter region of the NQO2 gene was a significant ($p = 0.038$) difference between patients with prolonged-type MAP psychosis and controls. This study suggests that the NQO2 gene polymorphism contributes to the aetiology of MAP-related psychosis in Japanese.

Introduction

Misuse of methamphetamine (MAP), the most abused drug in Japan, is a growing problem world-wide. Several lines of evidence suggest that genetic factors contribute to the vulnerability of substance abuse (Kendler 2001; Tsuang et al. 2001; Crabbe 2002; Uhl et al. 2002). Oxidative stress plays a role in the mechanisms of action of MAP: dopamine (DA) is released from the vesicular storage to the cytoplasm by administration of MAP, where DA can auto-oxidize to produce DA-quinones and additional reactive oxygen species, suggesting that DA-quinones might be implicated in the mechanism of action of MAP-induced neurotoxicity or psychosis in humans (Kita et al. 2003; Hashimoto et al. 2004).

The detoxification of quinones is catalyzed by a family of proteins designated as quinone oxidoreductase (NQOs). NQOs catalyze two-electron reduction to detoxify quinones competing with the one-electron reduction for the metabolism of quinones. In humans, genetic evidence indicates that the different forms of NQOs are encoded by four gene loci. Two of these gene loci have been identified as cytosolic NAD(P)H-quinone oxidoreductase 1 (NQO1) (Ross et al. 2000) and

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ISSN 1355-6215 print/ISSN 1369-1600 online/05/020145–04

DOI: 10.1080/13556210500123423

Taylor & Francis Group Ltd

NRH-quinone oxidoreductase 2 (NQO2) (Long and Jaiswal 2000). Quinones are highly reactive molecules and readily generate unstable semiquinones by reduction. Semiquinones undergo redox cycling in the presence of molecular oxygen and this leads to formation of highly reactive oxygen species (ROS). ROS and semiquinones cause oxidative stress. NQOs catalyze two-electron reduction competing with the one-electron reduction to detoxify quinones and their derivatives, and protect cells against damage associated with redox cycling and oxidative stress (Long and Jaiswal 2000; Ross et al. 2000). The insertion / deletion (I/D) polymorphism in the promoter region of the NQO2 gene has shown positive correlation with idiopathic Parkinson's disease (Harada et al. 2001), schizophrenia (Harada et al. 2003) and alcohol withdrawal symptoms (Okubo et al. 2003), although no difference was detected for the polymorphisms of the NQO1 gene and the other polymorphic loci of the NQO2 gene. Based on the role of oxidative stress in MAP-induced neurochemical changes, it is of great interest to examine association between polymorphisms of the NQO genes and MAP abusers. The purpose of the present study was to elucidate genetic vulnerability in Japanese MAP abusers associated with the polymorphisms of the NQO genes. In this study, we examined the association between the polymorphisms in the NQO1 gene (Pro187Ser) and the promoter region of the NQO2 gene and MAP abusers in Japan.

Materials and methods

The research was performed after obtaining approval from the ethics committees of each institute of Japanese Genetics Initiative for Drug Abuse (JGIDA), and all subjects provided written informed consent to the use of their DNA samples for this research. The subjects were 191 patients with MAP dependence and psychotic disorder meeting International Classification of Diseases version 10 (ICD-10) – Diagnostic Criteria for Research (DCR) criteria (F15.2 and F15.5) who were out-patients or in-patients of psychiatric hospitals of JGIDA, and 207 age-, gender- and geographical origin-matched normal controls were recruited mainly from medical staffs that had no past and family history of drug dependence or psychotic disorders (Table 1). All subjects were Japanese. Genomic DNA was extracted from whole blood obtained from the subjects. The patients were classified into three groups by some clinical features: (1) patients with MAP psychosis; (2) patients experienced or not experienced spontaneous relapse; and (3) patients with or without polysubstance abuse. Patients with MAP psychosis were also divided into two subgroups according to the duration of psychotic state: patients with prolonged-type MAP psychosis, whose psychotic state continued for more than 1 month; and patients with transient-type MAP psychosis, whose psychotic state improved within 1 month.

The polymorphism of a C to T substitution in exon 3 of the NQO1 gene (Pro187Ser) was identified by polymerase chain reaction (PCR)-restriction fragment length polymorphism (RFLP) using *Hinf* I (New England Biolabs Inc., Beverly, MA, USA). PCR primers were as follows: forward primer, 5′-TCCTCAGAGTGGCATTCTGC-3′ and reverse primer, 5′-TTCTCCTCATCCTGTACCTCT-3′; the annealing temperature was 58°C. The PCR product [240 base pairs (bp)] were cut by the restriction enzyme of *Hinf* I, producing two fragments of 215 and 25 bp for the C/C genotype, four fragments of 215, 180, 35 and 25 bp for the C/T genotype and two fragments of 180 and 35 bp for the T/T genotype. To genotype the polymorphism of I/D of 29 bp nucleotides in the promoter region of the NQO2 gene, PCR analysis was performed using the primers 5′-CTGCCTGGAAGTCAGCAGGGTC-3′ and 5′-CTCTTTACGCAGCGCGCCTAC-3′; the annealing temperature was 64°C. The PCR product size for homozygous genotype of insertion (I/I) was 291 bp and that for homozygous genotype of deletion (D/D) was 262 bp. Heterozygous genotype of insertion and deletion (I/D) made 310-bp heteroduplex DNA fragment in addition to 291-bp and 262-bp PCR products (Harada et al. 2001).

Differences in the distribution of genotypes between patients and controls were analysed using Fisher's exact probability test based on a two-sided p value. The comparison of mean age between the two groups was

Table 1. *Characteristics of the study population*

Characteristics	Controls	Abusers	p value
Number of subjects (male/female)	207 (161/46)	191 (151/40)	0.808*
Mean age, years (SD)	36.5 (11.9)	36.7 (10.6)	0.852†
MAP psychosis		147	
Transient		81	
Prolonged		60	
Unknown		6	
Spontaneous relapse			
Positive		61	
Negative		106	
Unknown		24	
Polysubstance abuse			
Yes		113	
No		52	
Unknown		26	

*Comparison of the male/female ratio between the two groups was performed by a Fisher's exact probability test. †Comparison of mean age between the two groups was performed by an unpaired t-test.

performed by an unpaired *t*-test (SPSS J for Windows 12.0.1 J, SPSS Japan Inc., Tokyo). Significance for the results was set at $p < 0.05$.

Results

The genotype distributions of NQO1 gene and NQO2 gene in patients and controls were in good agreement with the values expected from Hardy–Weinberg equilibrium. The genotype and allele frequencies for the polymorphism (Pro187Ser) of the NQO1 gene did not differ significantly between each subgroup of patients and controls (Table 2). The genotype and allele frequencies for I/D polymorphism in the promoter region of the NQO2 gene are summarized in Table 3. There was a significant difference between patients with prolonged-type MAP psychosis and controls ($p = 0.038$). In addition, there was a tendency to different genotype distribution of the NQO2 gene polymorphism in patients with MAP psychosis compared to controls ($p = 0.085$). No difference was detected in the genotype distribution between patients in the other subgroups and controls. The allele frequencies for I/D polymorphism in the promoter region of the NQO2 gene were not different between each subgroup of patients and controls. There was no gender difference between patients and controls (Table 1).

Discussion

We found that the I/D polymorphism in the promoter region of the NQO2 gene was associated with the prolonged type of MAP psychosis. It has been shown that the I/D polymorphism in the promoter region of the NQO2 gene was associated with genetic vulnerability to idiopathic Parkinson's disease (Harada et al. 2001) and schizophrenia (Harada et al. 2003). Furthermore, it has been reported that the I/D polymorphism in the promoter region of the NQO2 gene plays a role in the pathogenesis of alcoholism and alcohol withdrawal symptoms (Okubo et al. 2003). Therefore, our finding is of interest as MAP psychosis is similar to positive symptoms of schizophrenia (Sato et al. 1992).

Table 2. *Genotype and allele frequencies for the polymorphism (Pro187Ser) of the NQO1 gene*

		Genotype (%)				Allele (%)		
	n	CC	CT	TT	*p* value	C	T	*p* value (OR, 95% CI)
Controls	207	85 (41.1)	95 (45.9)	27 (13.0)		265 (64.0)	149 (36.0)	
Patients	191	69 (36.1)	97 (50.8)	25 (13.1)	0.577	235 (61.5)	147 (38.5)	0.510 (1.11, 0.83–1.48)
MAP psychosis	147	55 (37.4)	75 (51.0)	17 (11.6)	0.642	185 (62.9)	109 (37.1)	0.812 (1.05, 0.77–1.43)
Transient	81	36 (44.4)	35 (43.2)	10 (12.3)	0.886	107 (66.0)	55 (34.0)	0.645 (0.91, 0.62–1.34)
Prolonged	60	17 (28.3)	36 (60.0)	7 (11.7)	0.139	70 (58.9)	50 (41.1)	0.284 (1.27, 0.84–1.92)
Spontaneous relapse								
Positive	61	24 (39.3)	28 (45.9)	9 (14.8)	0.939	76 (62.3)	46 (37.7)	0.749 (1.08, 0.71–1.63)
Negative	106	34 (32.1)	60 (56.6)	12 (11.3)	0.206	128 (60.4)	84 (39.6)	0.383 (1.17, 0.83–1.64)
Polysubstance abuse								
Yes	113	41 (36.3)	53 (46.9)	19 (16.8)	0.556	135 (59.7)	91 (40.3)	0.306 (1.20, 0.86–1.67)
No	52	17 (32.7)	31 (59.6)	4 (7.7)	0.221	65 (62.5)	39 (37.5)	0.820 (1.07, 0.68–1.66)

Statistical analysis was performed by a Fisher's exact probability test (vs. control). OR, odds ratio; 95% CI, 95% confidence interval.

Table 3. *Genotype and allele frequencies for the insertion / deletion (I / D) polymorphism in the promoter region of the NQO2 gene*

		Genotype (%)				Allele (%)		
	N	I / I	I / D	D / D	*p* value	I	D	*p* value (OR, 95% CI)
Controls	207	123 (59.4)	74 (35.7)	10 (4.8)		320 (77.3)	94 (22.7)	
Patients	191	117 (61.3)	59 (30.9)	15 (7.9)	0.336	293 (76.7)	89 (23.3)	0.866 (1.13, 0.74–1.44)
MAP psychosis	147	93 (63.3)	40 (27.2)	14 (9.5)	0.085	226 (76.9)	68 (23.1)	0.928 (1.02, 0.72–1.46)
Transient	81	48 (59.3)	27 (33.3)	6 (7.4)	0.655	123 (75.9)	39 (24.1)	0.742 (1.08, 0.70–1.65)
Prolonged	60	40 (66.7)	13 (21.7)	7 (11.7)	0.038*	93 (77.5)	27 (22.5)	1.000 (0.99, 0.61–1.61)
Spontaneous relapse								
Positive	61	39 (63.9)	18 (29.5)	4 (6.6)	0.578	96 (78.7)	26 (21.3)	0.805 (0.92, 0.56–1.51)
Negative	106	64 (60.4)	32 (30.2)	10 (9.4)	0.229	160 (75.5)	52 (24.5)	0.619 (1.11, 0.75–1.63)
Polysubstance abuse								
Yes	113	68 (60.2)	36 (31.9)	9 (8.0)	0.477	172 (76.1)	54 (23.9)	0.769 (1.07, 0.73–1.57)
No	52	35 (67.3)	12 (23.1)	5 (9.6)	0.128	82 (78.8)	22 (21.2)	0.793 (0.91, 0.54–1.54)

Statistical analysis was performed by a Fisher's exact probability test (vs. control). *$p < 0.05$ vs. controls. OR, odds ratio; 95% CI, 95% confidence interval.

It has been reported that the expression of NQO2 in the D/D genotype of the NQO2 gene is lower than that in the I/I genotype (Harada et al. 2003), suggesting that insufficient NQO2 upregulation and/or activity might be vulnerable to Parkinson's disease (Harada et al. 2001) and schizophrenia (Harada et al. 2003). MAP is also known to cause oxidative stress and neurotoxicity, leading to the irreversible damages to the brain. Insufficient NQO2 activity could not provide enough neural protection against a large amount of DA-quinones derived from DA released by MAP. In this study, the percentage (11.7%) of D/D of the NQO2 gene in patients with prolonged-type MAP psychosis was approximately twofold higher than that (4.8%) of controls (Table 3). Therefore, it is likely that lower expression of NQO2 may contribute to the prolongation of MAP psychosis.

The homozygous T genotype of the NQO1 genes is a risk factor for breast cancer (Menzel et al. 2004), lung cancer (Xu et al. 2001) and leukemia (Naoe et al. 2000) because NQO1 activity was not detected in the T/T genotype (Traver et al. 1997). On the other hand, our study suggests that the polymorphism (Pro187Ser) of the NQO1 gene is not associated with MAP abusers in Japan. Furthermore, it has been shown that the NQO1 gene polymorphism (Pro187Ser) is not associated with Parkinson's disease (Harada et al. 2001) and schizophrenia (Hori et al. 2003) in the Japanese samples. The lack of association of the NQO1 gene in the present study does not necessarily mean that an investigation with another sample would give the same negative result. Frequency (1.6-3.7%) of the T/T genotype in Caucasian populations (Xu et al. 2001; Sarbia et al. 2003) is lower than that (13-17.2%) of the Japanese population (Harada et al. 2001; Hori et al. 2003), suggesting the ethnic difference between Japanese and Caucasians for the NQOI gene polymorphism (Pro187Scr).

In conclusion, the present findings suggest that NQO2, but not NQO1, may contribute to the aetiology of prolonged-type MAP psychosis in the Japanese population. The sample number in this study was relatively large, but not enough. Further studies are needed to elucidate genetic vulnerability to MAP abuse, paying more attention to clinical progress and state of the disease in a larger sample of Japanese. If replication studies are confirmed, the I/D polymorphism in the promoter region of the NQO2 gene would be the known specific mechanism by which genetic variation leads to a risk for the development of MAP-induced psychosis.

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American Journal of Medical Genetics Part B (Neuropsychiatric Genetics) 134B:90–92 (2005)

No Association of GSK3β Gene (*GSK3B*) With Japanese Schizophrenia

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Several lines of evidence indicate that glycogen synthase kinase-3β (GSK3β) is one of the candidates for schizophrenia-susceptibility factor. However, it has not been reported the association analysis between GSK3β gene (*GSK3B*) and Japanese schizophrenia based on linkage disequilibrium (LD). We provide an association analysis using relatively large samples (381 schizophrenia, and 352 controls) after determination of "tag single nucleotide polymorphisms (SNPs)." In this LD mapping, we selected and genotyped for eight polymorphisms (seven SNPs and one diallelic $(CAA)_n$ repeat), which covered the entire region of GSK3B, and determined two "tag SNPs." In the following association analysis using these two "tag SNPs," we could not find association with Japanese schizophrenia. Furthermore, we also include subgroup analysis considering age-at-onset and subtypes, neither could we find associations. Because our samples provided quite high power, these results indicate that GSK3B may not play a major role in Japanese schizophrenia.

KEY WORDS: linkage disequilibrium; tag SNP; association analysis

INTRODUCTION

Glycogen synthase kinase-3β (GSK3β) is one of the attractive candidate molecules for schizophrenia-susceptibility factor based on the following findings. (1) GSK3β is a key component of many signal transduction cascades including the phosphatidylinositol 3-kinase cascade and the Wnt cascade. (2) GSK3β is a critically important regulator of several transcriptional factors, and can influence the expression of numerous genes [Grimes and Jope, 2001]. (3) GSK3β levels were decreased in the prefrontal cortex of schizophrenia [Kozlovsky et al., 2000, 2001], and phosphorylation of GSK3β levels were also decreased in peripheral lymphocytes and brains of schizophrenia [Emamian et al., 2004]. (4) AKT1, one of the mediators of GSK3β, was reported to be associated with schizophrenia [Emamian et al., 2004].

Thus, we provide an association analysis based on linkage disequilibrium (LD) between the GSK3β gene (*GSK3B*) and Japanese schizophrenia.

MATERIALS AND METHODS

Subjects

Subjects for LD mapping consisted of 96 controls. For association analysis, total 381 patients with schizophrenia and 352 controls were genotyped. And the patients were divided into subgroups considering age-at-onset (AAO; early-onset schizophrenia (EOS) with onset younger than age 19) and subtypes of schizophrenia. The general characterization of these subjects and description of their psychiatric assessment are in Supplementary on line material. 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 and Nagoya University Graduate School of Medicine.

SNP Selection

For LD mapping, we selected seven SNPs and one diallelic $(CAA)_n$ repeat to make polymorphisms distributed appropriate intervals (Fig. 1). Then we determined "tag SNPs," which were the highest minor allele frequencies (MAFs) from "LD block," or which were independent from "LD block." More details are in Supplementary on line material.

Statistical Analysis

A detailed description can be seen in Supplementary on line material (see the online Supplementary Material at http://www.interscience.wiley.com/jpages/1552-4841/suppmat/index.html.).

RESULTS

For LD mapping, we genotyped eight polymorphisms for 96 controls. First, we evaluated the deviations from HWE for all polymorphisms. Each genotype frequency was in HWE. Next, we evaluated the pairwise LD matrices and determined "tag SNPs" from the LD blocks. One LD block was detected, and SNP6 was determined as a "tag SNP" for this block. Another SNP (SNP8) were in tight LD with this "LD block," however, the LD matrices among this "LD block" and SNP8 were not fit the criteria. Consequently, we determined two SNPs (SNP6 and SNP8) as "tag SNPs" (Fig. 2).

For association analysis, we expanded genotyping of these two "tag SNPs" for the rest samples. In this step, also genotype frequencies were in HWE. There were no significant associations between each "tag SNP" and schizophrenia. In the subgroup analysis considering AAO (EOS = 81), nor could we find associations between EOS and controls. And in another

This article contains supplementary material, which may be viewed at the American Journal of Medical Genetics website at http://www.interscience.wiley.com/jpages/1552-4841/suppmat/index.html.

Grant sponsor: Ministry of Education, Culture, Sports, Science and Technology; Grant sponsor: Ministry of Health, Labor and Welfare.

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Received 6 July 2004; Accepted 20 October 2004

DOI 10.1002/ajmg.b.30155

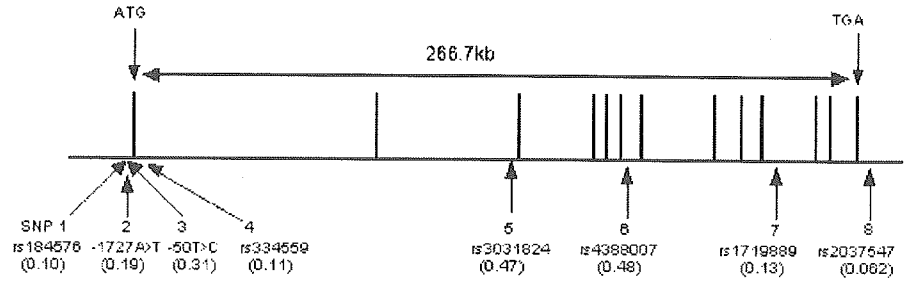

Fig. 1. Genomic structure of GSK3B with single nucleotide polymorphisms (SNPs) used in linkage disequilibrium (LD) mapping. Vertical bars represent exons. Numbers under arrows represent SNP ID. Parenthetic numbers represent minor allele frequencies (MAFs) of 96 controls.

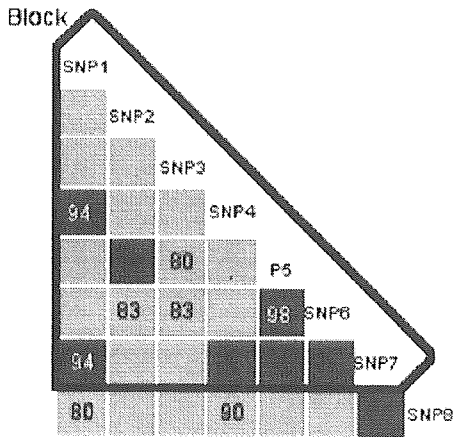

Fig. 2. LD mapping of GSK3B for control subjects. Numbers in box represent D' values after decimal point. D' values of 1.0 are not shown. The other information is described in Haploview's website.

subgroup analysis by subtypes, we did not analyze the association between catatonic type and controls because of small sample size. Neither could we find associations between each subtype (paranoid, disorganized, and residual type) and controls (Table I).

DISCUSSION

In this study, we could not find association of "tag SNPs" in GSK3B with Japanese schizophrenia in accordance with common disease-common variant (CD-CV) hypothesis [Reich and Lander, 2001].

This initial LD mapping using control samples showed that GSK3B was typical LD pattern. Although SNP8 and one LD block were in tight LD each other, SNP8 was not included in this LD block. This might be derived from low MAF of SNP8.

And P5, which was reported the positive association with "paranoid type" [Scassellati et al., 2004], was involved in this LD block. This indicates that P5 was represented by SNP6, which was the highest MAF in this LD block with the highest power for association analysis. Considering these polymorphisms in the LD mapping might be just markers [Scassellati et al., 2004], we did not genotype all of these, thereby avoiding redundant results.

Aside from this, the LD pattern among SNP2, SNP3, and P5 were different from the previous reports [Russ et al., 2001; Scassellati et al., 2004]. These discrepancies might be derived from the difference of population [Wall and Pritchard, 2003].

The power of our analysis was quite high, more than 80% for susceptibility gene whose genotype relative risk (GRR) (multiplicative model) set 1.34 (SNP6) and 1.73 (SNP8). And the power of "paranoid type" were more than 80% when GRR set 1.49 (SNP6).

Two points of caution must be noted in interpreting these negative results. (1) We must perform a systematic mutation

TABLE I. Association Analysis Between GSK3B and Japanese Schizophrenia Using Tag SNPs'

SNP ID	Samples	Subgroups[a]	N[b]	Genotype[c] M/M	M/m	m/m	*P*-value
SNP6	Control		352	103	180	69	
	Schizophrenia		381	100	203	78	0.66
		EOS	81	19	45	17	0.58
		Paranoid	136	35	73	28	0.74
		Disorganized	107	30	58	19	0.84
		Residual	87	24	48	15	0.78
SNP8	Control		352	311	40	1	
	Schizophrenia		381	341	38	2	0.69
		EOS	81	71	10	0	0.88
		Paranoid	136	122	14	0	0.91
		Disorganized	107	98	9	0	0.60
		Residual	87	76	9	2	0.16

[a]EOS, early onset of schizophrenia.
[b]N, numbers.
[c]M, major allele; m, minor allele.

search to detect "true" predisposing polymorphism. (2) Other candidate genes in this cascade must be studied in considering locus heterogeneity [Schork et al., 2001]. Recently, two controversial replications between AKT1, a mediator of GSK3β, and Japanese schizophrenia have been reported [Ikeda et al., 2004; Ohtsuki et al., 2004]. This concept might explain the discrepancy of these results, and suggest that combined analysis of this signaling cascade would be required for more concrete conclusions.

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American Journal of Medical Genetics Part B (Neuropsychiatric Genetics) 135B:5–9 (2005)

Research Articles

A Functional Glutathione *S*-Transferase P1 Gene Polymorphism Is Associated With Methamphetamine-Induced Psychosis in Japanese Population

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Several lines of evidence suggest that oxidative stress plays a role in the mechanisms of action of methamphetamine (MAP) in the human brain. Given the role of glutathione *S*-transferases (GSTs) in the protection against oxidative stress, genes encoding the GSTs have been considered as candidates for association studies of MAP abuse. This study was undertaken to investigate the role of the functional polymorphism of *GSTP1* gene exon 5 (Ile105Val) in the pathogenesis of MAP abuse. Genotyping for *GSTP1* gene polymorphism exon 5 (Ile105Val) in 189 MAP abusers and 199 normal controls was performed by polymerase chain reaction (PCR)-restriction fragment length polymorphism (RFLP). Association between *GSTP1* gene polymorphism and clinical features (prognosis of psychosis (transient-type and prolonged-type), spontaneous relapse (positive and negative), and poly-substance abuse) of MAP abusers was evaluated. Significant differences in the frequency of both alleles ($P = 0.026$, odds ratio: 1.70, 95% confidence intervals (CI) 1.06–2.72) and genotypes ($P = 0.029$) between MAP abusers and controls were detected. In particular, a significant difference in both genotype frequency ($P = 0.013$) and allele frequency ($P = 0.014$, odds ratio: 1.84, 95% CI 1.13–2.97) between MAP abusers with psychosis (transient-type and prolonged-type) and controls was detected. Our findings suggest that the polymorphism (Ile105Val) on exon 5 of the *GSTP1* gene may contribute to a vulnerability to psychosis associated with MAP abuse in Japanese population.

KEY WORDS: methamphetamine; psychosis; drug abuse; genetic factor; polymorphism

INTRODUCTION

Abuse of methamphetamine (MAP) is a growing problem worldwide. Some lines of evidence suggest that both environmental and genetic factors might contribute to drug abuse vulnerability [Merikangas et al., 1998; Kendler et al., 2000; Rawson et al., 2002; Uhl et al., 2002; Cami and Farre, 2003]. It is well known that MAP induces a strong psychological dependence, and that repeated further consumption of MAP results in psychotic states, the symptoms of which resemble those of the paranoid type of schizophrenia [Sato et al., 1983, 1992].

Positron emission tomography (PET) imaging studies of the brains of MAP abusers have demonstrated that the density of dopamine (DA) transporters is significantly decreased in the caudate/putamen of MAP abusers [Sekine et al., 2001; Volkow et al., 2001]. Such findings suggest that the long-term use of MAP leads to the damage of dopaminergic neurons in the human brain. It has been shown that MAP-induced neurotoxicity in the brain requires the involvement of striatum DA and

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Received 10 August 2004; Accepted 22 November 2004
DOI 10.1002/ajmg.b.30164

also involves mechanisms associated with oxidative stress, further suggesting that oxidative stress in dopaminergic pathways might be implicated in MAP-induced neurotoxicity [Cadet et al., 2003]. There are a number of papers demonstrating the neuroprotective effects of glutathione or its related compounds on MAP- or DA-induced neurotoxicity [Choi et al., 2002; Shimizu et al., 2002; Fukami et al., 2004; Hashimoto et al., 2004]. In addition, it is also well known that dopaminergic pathways in the mesocorticolimbic systems can play an important role in drug reward [Kalivas, 2002]. Therefore, polymorphisms in genes that regulate dopaminergic pathways may contribute to interindividual differences as regards a vulnerability to drug abuse [Koob and Le Moal, 1997].

The glutathione *S*-transferases (GSTs: EC 2.5.1.18) belong to a family of multifunctional enzymes that catalyze the conjugation of reduced glutathione with electrophilic groups of a wide variety of compounds including carcinogens, environmental contamination, and products of the oxidative process [Mannervik, 1985; Smythies and Galzigna, 1998; Hayes and Strange, 2000]. Because of their important role in the cellular defense against oxidative stress, GSTs are of interest in the context of association studies of MAP abuse. The genes encoding three classes of GSTs, i.e., GSTM (mu, chromosome 1p13.3), GSTP (pi, chromosome 11q13), and GSTT1 (theta, chromosome 22q11.2), are known to be polymorphic [Watson et al., 1998; Stucker et al., 2002; De Roos et al., 2003; Kelada et al., 2003; Wang et al., 2003]. Recently, we reported an association between *GSTM1* gene deletion and female MAP abusers, suggesting that *GSTM1* gene deletion may contribute to a vulnerability to MAP abuse in Japanese subjects [Koizumi et al., 2004]. Furthermore, it has been reported that genetic polymorphisms of GSTP1 exon 5 (rs947894, Ile105Val (A > G)) and exon 6 (rs1799811, Ala114Val (C > T)) have functional relevance to the GST gene product resulting in reduced GST enzyme activity (~30%) [Board et al., 1989; Zimniak et al., 1994; Ali-Osman et al., 1997; Watson et al., 1998]. Taken together, such findings appear to suggest that individuals with these variant GSTP1 genotypes which result in reduced GSTP1 enzymatic activity may be at greater risk of MAP abuse. In order to verify the potential role of the *GSTP1* gene in the pathogenesis of MAP abuse, we analyzed a polymorphism of the *GSTP1* gene in subjects with a diagnosed MAP-related disorder.

METHODS

This study was performed after obtaining the approval of the ethics committees of each affiliated institute of the Japanese Genetics Initiative for Drug Abuse (JGIDA). All subjects provided written informed consent for the use of their DNA samples for this study. The subjects were 189 patients (149 males and 40 females, age: 36.9 ± 11.9 years (mean ± SD), age range: 18–69 years) with MAP dependence and a psychotic disorder meeting the ICD-10-DCR criteria (F15.2 and F15.5) who were outpatients or inpatients of psychiatric hospitals of the JGIDA (Table I). The control subjects were 199 age-, gender-, and geographical origin-matched normal controls (157 males and 42 females, age: 37.2 ± 10.5 years (mean ± SD), age range: 19–73 years), the majority of whom were with no past history and no family history of drug dependence or psychotic disorders. Diagnoses were made by two trained psychiatrists by interview and available information including hospital records. Patients were excluded if they had a clinical diagnosis of schizophrenia, another psychotic disorder, or an organic mental syndrome as reported previously [Ujike et al., 2003]. All subjects were Japanese, born and living in restricted areas of Japan including northern Kyushu, Setouchi, Tyukyou, Toukai, and Kantou.

The patients were divided into subgroups by some characteristic clinical features (Table I). The patients were divided by poly-substance abuse status, 55 patients abuse MAP only in their lifetime, and 116 patients abuse some other drugs besides MAP in present or past. Organic solvent was most frequently abused besides MAP, followed by marijuana. Cocaine and heroine were rarely abused in the present study. Prognosis of MAP psychosis was various among patients, and some patients showed continuous psychotic symptoms even after MAP discontinuance as previously reported [Sato et al., 1983, 1992]. Therefore, patients were divided into two categories of prognosis, transient-type and prolonged-type, based on duration of psychotic state after MAP discontinuance. Thus, patients with transient-type whose psychotic symptoms improves within 1 month after discontinuance of MAP consumption and beginning of treatment with antipsychotic drugs, and those with prolonged-type whose psychosis continues for more than 1 month even after discontinuance of MAP consumption and beginning of treatment. In this study, patients with transient- and prolonged-types of MAP psychosis were 94 and 65, respectively (Table I). It has been well documented that once MAP psychosis has developed, patients in remission state becomes reliable to spontaneous relapse without re-consumption of MAP [Sato et al., 1983, 1992]. It is postulated that sensitization phenomenon induced by repeated consumption of MAP should be developed in the brain of MAP psychosis patients which result in neural basis for enhanced susceptibility to relapse. Therefore, the patients were divided into two groups according to presence or absence of spontaneous relapse. The patients with and without spontaneous relapse were 62 and 111, respectively (Table I).

Two polymorphisms on exon 5 and exon 6 of the *GSTP1* gene have previously been reported. We analyzed exon 5 (rs947894, Ile105Val) of the *GSTP1* gene in this study, since no minor

TABLE I. Characteristics of Control Subjects and MAP Abusers

Variable	Controls	Abusers	*P*-value
Sex, M/F	157/42	149/40	0.989[a]
Age, mean ± SD, years	37.2 ± 10.5 (19–73)	36.9 ± 11.9 (18–69)	0.813[b]
Prognosis of psychosis			
Transient type		94	
Prolonged type		65	
Spontaneous relapse			
Positive		62	
Negative		111	
Poly-substance abuse			
No		55	
Yes		116	

[a]The comparison between two groups was performed using the χ^2 test.
[b]The comparison between two groups was performed using the *t*-test.

allele frequency of the polymorphism of exon 6 (rs1799811, Ala114Val) was detected among Japanese normal subjects [Ishii et al., 1999]. Genotyping for this gene was performed by PCR-RFLP analysis. The polymorphic site in exon 5 (Ile105-Val) was amplified as reported previously [Wang et al., 2003]. The primers of exon 5 of the *GSTP1* gene were GSTP1-5F (5′-GTAGTTTGCCCAAGGTCAAG-3′) and GSTP1-5R (5′-AGC-CACCTGAGGGGTAAG-3′). After performing PCR, a 433 bp DNA fragment was amplified for GSTP1 exon 5, followed by 2 hr digestion with BsmA I (New England Biolabs, Inc., Beverly, MA). The fragments were separated on 2% agarose gel stained with ethidium bromide. The wild-type (A/A), heterozygous genotype (A/G), and mutant genotype (G/G) yielded two bands (328 and 105 bp), four bands (328, 222, 106, and 105 bp), and three bands (222, 106, and 105 bp), respectively.

The differences between groups were evaluated by Fisher's exact test. The odds ratio and 95% confidence intervals (CI) between two variables were calculated as an estimate of risk. Differences were considered significant at $P < 0.05$.

RESULTS

The frequencies of allele and genotypes in MAP abusers and controls are shown in Table II. The genotype distribution in both MAP abusers and controls was in the Hardy–Weinberg equilibrium. The differences in both genotype frequency ($P = 0.029$) and allele frequency ($P = 0.026$) between MAP abusers and controls were found to be significant (Table II). The frequency of carrying the G allele in MAP abusers was significantly higher ($P = 0.026$, odds ratio: 1.70, 95% CI 1.06–2.72) than that of controls.

Next, we examined the association between the clinical features of MAP abusers (i.e., prognosis of psychosis, spontaneous relapse, and poly-substance abuse) and normal controls. A significant difference in both genotype frequency ($P = 0.013$) and allele frequency ($P = 0.014$, odds ratio: 1.84, 95% CI 1.13–2.97) between MAP abusers with psychosis (transient-type and prolonged-type) and controls was detected (Table II). There was a significant difference in genotype frequency ($P = 0.045$) between MAP abusers with transient-type psychosis and controls, and was a trend toward difference in allele frequency ($P = 0.052$, odds ratio: 1.75, 95% CI 1.01–3.06) between MAP abusers with transient-type psychosis and controls. There was also a significant difference in both genotype frequency ($P = 0.028$) and allele frequency ($P = 0.039$, odds ratio: 1.96, 95% CI 1.07–3.59) between MAP abusers with prolonged-type psychosis and controls. Furthermore, a significant difference in terms of both genotype frequency ($P = 0.009$) and allele frequency ($P = 0.009$, odds ratio: 2.00, 95% CI 1.19–3.35) between MAP abusers with negative spontaneous relapse and controls was detected (Table II). Moreover, there was a trend toward difference in both genotype frequency ($P = 0.052$) and allele frequency ($P = 0.053$, odds ratio: 1.70, 95% CI 1.00–2.88) between MAP abusers with poly-substance abuse and controls (Table II).

DISCUSSION

Our findings suggest that a functional polymorphism (Ile105Val) on exon 5 of the *GSTP1* gene may contribute to MAP abuse vulnerability in Japanese subjects. Since a polymorphism (Ile105Val) on exon 5 has been shown to be of functional significance in terms of enzyme activity [Zimniak et al., 1994; Watson et al., 1998], individuals with the G allele (valine) would be expected to have decreased GST detoxification. Based on the role of GSTs in the antioxidant system preventing MAP-induced neurotoxicity, variant *GSTP1* genes might lead to an excess of metabolic products (e.g., DA-quinone) of the oxidative process induced by the administration of MAP, and may furthermore lead to MAP-induced neurotoxicity in the brain, including damage of the dopaminergic neurons, as compared to the products associated with the A allele (isoleucine) of *GSTP1* gene. We also found that the frequency of the G allele in MAP abusers with psychosis (transient-type and prolonged-type) was significantly higher than that of controls, suggesting that this *GSTP1* gene polymorphism may be associated with MAP-induced psychosis in Japanese subjects. Thus, it appears to be the case that the GSTP1 polymorphism (Ile105Val) on exon 5 may contribute to a susceptibility to MAP-induced psychosis among Japanese subjects. In contrast, we found an association between GSTP1 polymorphism (Ile105Val) and negative spontaneous relapse, whereas no association between this polymorphism and positive spontaneous relapse was detected. Taken together, it seems that GSTP1 polymorphism (Ile105Val) may be implicated in MAP-induced psychosis, but not spontaneous relapse, although further studies using a large sample are necessary.

It has been suggested that DA-quinones synthesized by auto-oxidation of DA might play a role in MAP-induced neurotoxicity in the brain, and that glutathione and GST might play a role in the detoxification against DA-quinone induced neurotoxicity [Smythies and Galzigna, 1998; LaVoie and Hastings, 1999; Whitehead et al., 2001; Shimizu et al., 2002; Asanuma et al., 2003]. Thus, DA auto-oxidation results in the formation of DA-quinones, which readily participate in nucleophilic addition reactions with sulfhydryl groups on free cysteine, glutathione, or cysteine found in proteins including DA transporters [Smythies and Galzigna, 1998;

TABLE II. Genotype and Allele Frequencies of the GSTP1 Exon 5 Gene Polymorphism in Controls and MAP Abusers

Ile105Val (A > G)		Genotype				Allele		
rs947894	n	AA	AG	GG	*P*	A	G	*P*
Control	199	167 (83.9%)	32 (16.1%)	0 (0%)		366 (92.0%)	32 (8.0%)	
Abuser	189	144 (76.2%)	41 (21.7%)	4 (2.1%)	0.029*	329 (87.0%)	49 (13.0%)	0.026*
Prognosis of psychosis	159	119 (74.8%)	36 (22.6%)	4 (2.5%)	0.013*	274 (86.2%)	44 (13.8%)	0.014*
Transient	94	71 (75.5%)	21 (22.3%)	2 (2.1%)	0.045*	163 (86.7%)	25 (13.3%)	0.052
Prolonged	65	48 (73.8%)	15 (23.1%)	2 (3.1%)	0.028*	111 (85.4%)	19 (14.6%)	0.039*
Spontaneous relapse								
Positive	62	50 (80.6%)	11 (17.7%)	1 (1.6%)	0.255	111 (89.5%)	13 (10.5%)	0.463
Negative	111	81(73.0%)	27 (24.3%)	3 (2.7%)	0.009**	189 (85.1%)	33 (14.9%)	0.009**
Poly-substance abuse								
No	55	44 (80.0%)	9 (16.4%)	2 (3.6%)	0.065	97 (88.2%)	13 (11.8 %)	0.254
Yes	116	87 (75.0%)	28 (24.1%)	1 (0.9%)	0.052	202 (87.1%)	30 (12.9%)	0.053

*$P < 0.05$.
**$P < 0.01$ as compared to control (Fisher's exact test).

Whitehead et al., 2001]. Based on the known role of GSTs in the process of antioxidant defense, we considered the possibility that MAP abusers with the G allele of GSTP1 polymorphism were more susceptible to MAP-induced psychosis or to a spontaneous relapse of MAP abuse. In this study, we found significant differences in the distribution of genotype and allele frequencies between MAP abusers with psychosis and controls. Furthermore, we found a significant difference between MAP abusers with negative spontaneous relapse and controls. Taken together, it is likely that the polymorphism (Ile105Val) on exon 5 of the *GSTP1* gene could be a risk factor for the development of MAP-induced psychosis in Japanese subjects.

It is reported that the frequency (18%) of the G allele in Asians such as Taiwanese is lower than that in African-American (42%) and European-American (33%) [Watson et al., 1998]. The frequency (8%; our study) of the G allele in Japanese control subjects is significantly ($\chi^2 = 13.3$, $P = 0.0003$) lower than that (18%) of Taiwanese, suggesting the ethnic difference between Asians and European- and African-Americans for the polymorphism (Ile105Val) on exon 5 of GSTP1. Therefore, it may be of interest to examine the association between the *GSTP1* gene polymorphism and methamphetamine abusers in European- and African-Americans. If replication studies are confirmed, the polymorphism (exon 5 Ile105Val) of GSTP1 would be only the known specific mechanism by which genetic variation leads to a risk for the development of MAP-induced psychosis. Interestingly, our recent PET study demonstrated that the antioxidant *N*-acetyl-L-cysteine (a precursor for glutathione synthesis) could attenuate significantly the reduction of DA transporter in monkey striatum after repeated administration of MAP [Hashimoto et al., 2004]. In addition, we reported that *N*-acetyl-L-cysteine attenuated hyperlocomotion, development of sensitization, and neurotoxicity after administration of MAP [Fukami et al., 2004], suggesting that *N*-acetyl-L-cysteine would be a suitable drug for treatment of MAP abuse. As described in "Introduction," it is likely that endogenous antioxidant glutathione plays a role in the behavioral changes and neurotoxicity associated with MAP abuse. Taken together, our findings may shed light on some of the neurobiological mechanisms and pathways that lead to the development of MAP abuse, and could thereby facilitate the development of novel treatments and prevention strategies for MAP abuse.

In conclusion, our findings indicate that a polymorphism (exon 5 Ile105Val) of the *GSTP1* gene may contribute to a vulnerability to MAP abuse among Japanese subjects. Furthermore, it is likely that this polymorphism (exon 5 Ile105Val) of the *GSTP1* gene could be a risk factor for the development of MAP-induced psychosis in Japanese subjects.

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Association Analysis of Chromosome 5 $GABA_A$ Receptor Cluster in Japanese Schizophrenia Patients

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Background: *Several investigations suggest that abnormalities in γ-amino butyric acid (GABA) neurotransmission systems may be related to the pathophysiology of schizophrenia. A $GABA_A$ receptor gene cluster on 5q31-35 (β2 [GABRB2], α6 [GABRA6], α1 [GABRA1], and γ2 [GABRG2] subunit genes) is one of the most attractive candidate regions for schizophrenia susceptibility.*

Methods: *We performed 1) systematic polymorphism search of GABRB2, GABRA6, and GABRA1, in addition to our colleague's study of GABRG2; 2) evaluation of linkage disequilibrium (LD) within this cluster with 35 single nucleotide polymorphisms (SNPs); 3) "selection of haplotype-tagging (ht) SNPs"; and 4) two-stage association analysis that comprised first-set screening analysis of all htSNPs (288 Japanese schizophrenia patients and 288 control subjects) and second-set replication analysis of the positive htSNPs (901 schizophrenic patients and 806 control subjects).*

Results: *In the first-set scan, we found a significant association of two htSNPs in GABRA1, but no association of GABRB2, GABRA6, and GABRG2. In the following second-set analysis, however, we could not confirm these significant associations.*

Conclusions: *These results indicate that this gene cluster may not play a major role in Japanese schizophrenia. They also raised an alert with regard to preliminary genetic association analysis using a small sample size.*

Key Words: Linkage disequilibrium, haplotypes, single nucleotide polymorphism, multiple testing

Gamma-amino butyric acid (GABA) is a major inhibitory neurotransmitter in the mammalian central nervous system. Recently, abnormalities in the GABA neurotransmission system have been considered to be a possible factor related to the pathophysiology of schizophrenia, on the basis of the following findings: 1) the alternation of GABA neurons in the prefrontal cortex of schizophrenia patients might contribute to cognitive dysfunction, one of the main features of schizophrenia (Benes and Berretta 2001; Blum and Mann 2002; Weinberger et al 1986); and 2) GABA has an important role in neurodevelopment (Carlsson et al 2001; Owens and Kriegstein 2002), the abnormality of which has been hypothesized in schizophrenia (Weinberger 1995).

The formation of $GABA_A$ receptor requires co-expression of α, β, and γ subunit genes that also form α-β-γ clusters on the same chromosomes (Russek 1999): 4p12, 5q31-35, and 15q11-13. Among them, a cluster on 5q31-35 composed of β2 (GABRB2), α6 (GABRA6), α1 (GABRA1), and γ2 (GABRG2) subunit genes is important because the products of genes of this cluster are abundant in the brain (McKernan and Whiting 1996; Whiting et al 1999) and play key roles in the mechanism of psychotropic drugs, including anxiolytics, anticonvulsants, and hypnotics. Multiple whole genome linkage studies of schizophrenia showed a linkage in 5q31-35 (DeLisi et al 2002; Gurling et al 2001; Kendler et al 2000; Levinson et al 2000; Lewis et al 2003; Paunio et al 2001; Sklar et al 2004) close to the location of the GABA gene cluster. Thus, the 5q cluster genes are attractive candidates for schizophrenia susceptibility. Recently, more interest has been focused on this cluster because a positive association was reported between GABRB2 and schizophrenia in Han Chinese (Lo et al 2004).

We previously performed a systematic polymorphism search and association analysis on the basis of linkage disequilibrium (LD) of GABRG2 in which we found no association between single nucleotide polymorphisms (SNPs) in GABRG2 and schizophrenia in Japanese patients (Nishiyama et al 2005).

In the present study we expanded this strategy to the $GABA_A$ receptor subunit gene cluster on 5q. After a systematic polymorphism search in this region, we evaluated LD with 35 SNPs and selected 21 "haplotype-tagging (ht) SNPs' with relatively strict criteria. We included two-stage association analyses with a different panel of samples, in which the significant htSNPs in the first-set screening analysis were further assessed in the second-set replication analysis. This strategy is powerful for genetic association analysis from the viewpoints of "htSNP" selection and the correction of multiple testing. Thus, the results might be able to reliably rule out type I and type II errors.

Methods and Materials

Subjects

The subjects for polymorphism search were 96 patients with various psychiatric disorders (37 schizophrenia, 27 bipolar I disorder, 2 major depressive disorder, 10 obsessive-compulsive disorder, 10 panic disorder, and 10 amphetamine-related disorder). For the evaluation of LD, 96 control subjects were used. In the first-set screening analysis, 288 patients with schizophrenia (148 men and 140 women; 39.6 ± 14.0 years [mean age ± SD]) and 288 control subjects (150 men and 138 women; 33.6 ± 14.0 years) were genotyped. In the second-set replication analysis, a different panel of samples was used that consisted of 901 patients (482 men and 419 women; 49.2 ± 15.0 years) and 806 control subjects (403 men and 405 women; 40.0 ± 14.1 years). All subjects were unrelated to each other and ethnically Japanese.

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 review of medical records. All healthy control subjects, about one-half of whom

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Received November 15, 2004; revised April 21, 2005; accepted May 2, 2005.

0006-3223/05/$30.00
doi:10.1016/j.biopsych.2005.05.002

were hospital staff or medical students and one-half recruited from the general population, were also psychiatrically screened on the basis of unstructured interviews. 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, Okayama University, and Nagoya University.

SNP Identification

Genomic DNA was extracted from peripheral blood of all subjects. Primer pairs were designed with information from the GenBank sequence (accession number: NM-023133.11) and 42 amplified regions that covered all the coding regions, the branch sites, and 5'-flanking regions, which are 500 base pair (bp) upstream from the initial exons of GABRB2, GABRA6, and GABRA1. For GABRG2, because we had already performed a polymorphism search in the coding regions, we added only the 5'-flanking regions 500 bp upstream from the initial exon. A more detailed description of methods can be seen in a previous paper (Suzuki et al 2003). Sequences of primer pairs are available on request.

SNP Selection

For the evaluation of LD, we included SNPs from databases (dbSNP, NCBI, Bethseda, Maryland; and Celera Discovery Systems, Rockville, Maryland) and other papers (Lo et al 2004; Nishiyama et al 2005) in addition to the SNPs we detected so that the SNPs were nearly evenly distributed. We excluded the minor allele frequencies (MAF) of SNPs less than .1 (Figure 1; Table 1).

First, we determined an "LD block" to be a region in which all pairwise D' values are not lower than .8, with the Genotype2LDblock v0.2 software (Zhang and Jin 2003). Next, "htSNPs" were selected within each "LD block" for 90% haplotype coverage with SNPtagger software (Ke and Cardon 2003). This program requires estimated haplotypes as input, for which we used PHASE version 2.1 (Stephens and Donnelly 2003; Stephens et al 2001). Single nucleotide polymorphisms that might have functional effects (i.e., SNPs in exons, untranslated regions and promoter regions) were selected preferentially, because they were considered potential candidates for predisposing factors.

SNP Genotyping

For rapid genotyping of SNPs, we used TaqMan assays (Applied Biosystems, Foster City, California), restriction fragment length polymorphism (RFLP) assays, and primer extension methods with denaturing high-performance liquid chromatography. The other SNPs were genotyped by the direct sequencing method. In particular, the genotyping of SNP20 and SNP24, which were positive SNPs in the first-set screening analysis, was done with 192 randomly selected samples (96 cases and 96 control subjects) with direct sequencing to check for genotyping error. TaqMan probes and Universal PCR Master Mix were obtained from Applied Biosystems. A 5-μL total reaction volume was used and, after PCR, the allelic specific fluorescence was measured on ABI PRISM 7900 Sequence Detector Systems (Applied Biosystems). The RFLP assays and primer extension methods were described in greater detail previously (Suzuki et al 2003). Detailed information, including primer sequences, is also available on request.

Statistical Analysis

Genotype deviation from the Hardy-Weinberg equilibrium (HWE) was evaluated by χ^2 test (SAS/Genetics, release 8.2, SAS Institute, Cary, North Carolina).

In the first-set screening analysis, marker-trait association was evaluated allele/genotype-wise with the Fisher exact test (SPSS 10.0J, SPSS Japan, Japan) and haplotype-wise with the program COCAPHASE 2.403 (Dudbridge 2003). The COCAPHASE program performs log-likelihood ratio tests under a log-linear model for global p value. To estimate haplotype frequencies of "htSNP" combinations in each LD block, the expectation-maximization algorithm was used. Rare haplotypes found in less than 3% of both cases and control subjects were excluded from association analysis to provide greater sensitivity and accuracy when the effect is seen in common haplotypes.

In the first-set screening analysis, we also used a recently developed software program, SNPSpD, which is able to reflect the correlation of markers (LD) on corrected p values, to control inflation of the type I error rate (Nyholt 2004).

In the second-set replication analysis, the association was also evaluated with identical allele- and genotype-wise methods, as described in this section.

Power calculation was performed with a statistical program prepared by Ohashi (Ohashi et al 2001). We estimated the power for our sample size under a multiplicative model of inheritance.

The significance level for all statistical tests was .05.

Results

We identified 6 SNPs in GABRB2, 9 SNPs in GABRA6, and 11 SNPs in GBARA1 (Table 1); however, we could not detect any

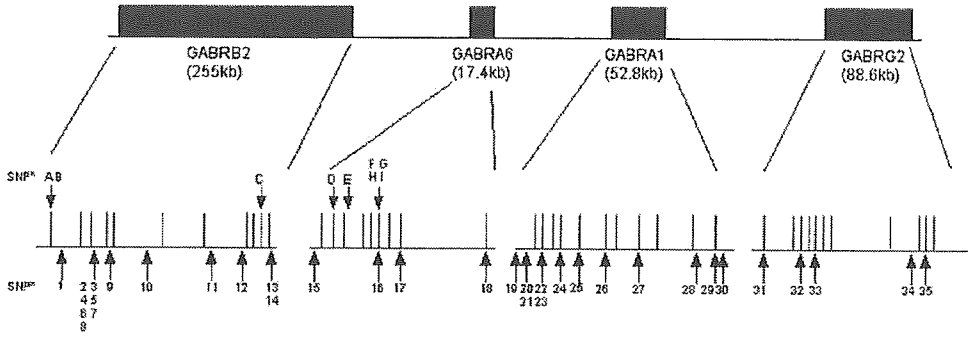

Figure 1. Overview of γ-amino butyric acid$_A$ (GABA$_A$) receptor gene cluster on 5q and individual gene structures. GABRB2, β2; GABRA6, α6; GABRA1, α1; and GABRG2, γ2. *shows single nucleotide polymorphism (SNP) ID in Table 1. Vertical bars represent exons.

Table 1. SNPs in Polymorphism Search, LD Mapping, and Association Analyses

Gene Symbol	SNP	ID	LD Block	LD Mapping: Identified SNP	LD Mapping: Selected SNP from Database	MAF (%)	Genotype Distribution: M/M SCH	M/M CON	M/m SCH	M/m CON	m/m SCH	m/m CON	p value[a]: (Allele)	p value[a]: (Genotype)	Corrected p value[b] (Genotype)
GABRB2	A			rs2229944		1.0									
	B			1227C > T		2.1									
		1[c]	I		rs253017	41.1	84	87	149	129	55	72	.409	.153	
		2[c]			rs187269	16.7	202	199	75	80	11	9	1.000	.827	
		3			rs252944	15.6									
		4			rs194072	15.6									
		5			rs1816072	38.0									
		6			rs1816071	31.3									
		7	II		rs6891988	16.7									
		8[c]			rs6556547	15.6	202	199	74	80	12	9	1.000	.690	
		9[c]		rs2303055		18.2	182	170	94	100	12	18	.202	.414	
		10[c]	III		rs1363697	27.6	152	141	116	118	20	29	.216	.352	
		11[c]			rs2962407	41.1	96	77	134	140	58	71	.0669	.177	
		12[c]	IV		hCV1703405	35.9	114	107	135	136	39	45	.467	.707	
	C			−141G > A		3.6									
		13[c]		rs3816596		47.4	63	73	154	147	71	68	.444	.624	
		14		−218-387G > A		11.4									
GABRA6		15	V		rs3811995	33.3									
	D			94G > A		<1.0									
	E			225 + 16G > A		<1.0									
	F			276G > A		<1.0									
	G			383A > G		<1.0									
	H			447-20G > A		<1.0									
	I			rs3811993		<1.0									
		16		673-121T > C		32.3									
		17[c]		1005C > G		32.3	121	141	141	123	26	24	.183	.242	
		18		rs3219151		32.3									
GABRA1		19	VI		hCV478645	39.6	117	110	140	144	31	34	.581	.831	
		20		−371-471C > T		42.2	91	123	154	128	43	37	.0249	.0213	.41
		21[c]	VII	−371-181A > G		35.9	123	117	122	126	43	45	.626	.870	
		22[c]	VIII	rs12658835		18.2	162	159	113	114	13	15	.784	.929	
		23[c]		rs4608967		18.2	170	160	102	103	16	25	.197	.323	
		24[c]		74 + 9A > T		17.7	214	242	71	43	3	3	.00830	.00862	.166
		25[c]		rs1129647		25.5	141	148	125	123	22	17	.472	.659	
		26		188-42C > T		22.4									
		27		rs11135172		21.9									
		28		rs22790720		42.2									
		29[c]		rs998754		40.6	70	68	151	158	67	62	.860	.823	
		30[c]		rs2290733		13.0	191	188	86	86	11	14	.709	.868	
GABRG2		31[c]	IX		rs2268583	26.6	152	155	113	114	23	19	.691	.816	
		32			rs11135176	26.0									
		33[c]			rs211037	38.5	85	84	152	142	51	62	.515	.497	
		34	X		rs211015	44.3									
		35[c]			rs211014	42.7	64	80	139	128	85	80	.216	.317	

SNP, single nucleotide polymorphism; LD, linkage disequilibrium; MAF, minor allele frequency of 96 subjects; M, major allele; m, minor allele; SCH, schizophrenic patients; CON, control subjects.
[a] *p* value from Fisher exact test.
[b] calculated using SNPSpD software.
[c] htSNPs for association analyses.

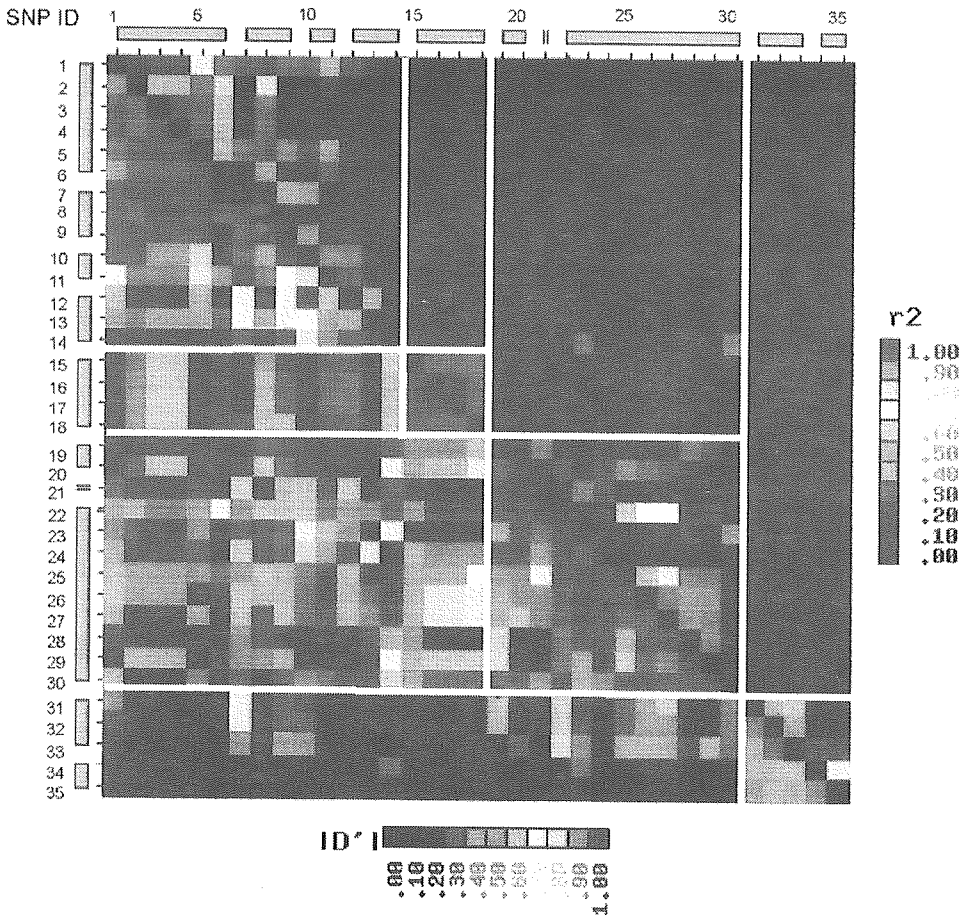

Figure 2. Linkage disequilibrium (LD) mapping; LD block are shown both top and left of the diagram. Top right triangles show r^2, bottom left triangles show D'. SNP, single nucleotide polymorphism.

functional SNPs such as non-synonymous SNPs. For GABRG2, we could not detect any SNPs within 500 bp upstream of the initial exon.

We genotyped all SNPs for 96 control subjects, because 96 samples were enough to measure LD among SNPs (Reich et al 2001). First, we evaluated the deviations from HWE for all SNPs and found the genotype frequencies to be consistent with HWE. Next, we evaluated pairwise LD between each SNP with MAF > .1, defined "LD blocks", and selected "htSNPs" for association analyses. Consequently, 21 "htSNPs" were selected (Figure 2; Table 1). We genotyped these "htSNPs" for 288 patients with schizophrenia and the remaining 192 control subjects (total 288 control subjects) for the first-set screening analysis. In this step, genotype frequencies were again in HWE.

The SNPs in GABRA6 were in almost perfect LD, and SNP17 was the only "htSNP" among SNPs in GABRA6; thus, we did not perform haplotypic analysis.

For genotype- and allele-wise association, only SNP20 and SNP24 were significantly associated with schizophrenia (genotype-wise p values: .0213, .00862 for SNP20 and SNP24, respectively; allele-wise p values: .0249, .00830; Table 1); however, p values corrected with the SNPSpD software did not reach the significant level (cluster-wide, the effective number of independent loci is 19.25; the experiment-wide significance threshold required that the type I error rate be kept at .05:.002597; Table 1). Furthermore, haplotype-wise analysis did not show significance with any haplotypes constructed from the combination of htSNPs in each LD block (Table 2). We also confirmed our negative results for GABRG2.

To obtain a conclusive result of the positive association in GABRA1, we conducted a second-set replication analysis of these two htSNPs with a larger and different panel of samples. No significant association could be confirmed (Table 3).

We also included a power calculation for second-set replication analysis. We obtained power of more than 80% to detect

Table 2. Global Haplotypic Analysis

Gene Symbol	Block	Combination of SNPs	Global p Values
GABRB2	I	SNP1–2	.710
	II	SNP8–9	.447
	III	SNP10–11	.151
	IV	SNP12–13	.633
GABRA1	VI	SNP19–20	.0840
	VIII	SNP22–23–24–25–29–30	.0508
GABRG2	IX	SNP31–33	.298

SNP, single nucleotide polymorphism.

Table 3. Second-Set Association Analysis of Two Positive htSNPs in First-Set Scan

SNP ID	Phenotype	Number	Genotype Distribution			*p* Values		Power Calculation (GRR)
			M/M	M/m	m/m	(Genotype)	(Allele)	
SNP20	SCZ	901	337	429	135	.792	.503	1.22
	CON	806	312	381	113			
SNP24	SCZ	901	709	179	13	.848	.703	1.34
	CON	806	639	158	9			

htSNP, haplotype-tagging single nucleotide polymorphism; M, major allele; m, minor allele; GRR, genotype relative risk to obtain 80% power; SCZ, schizophrenic patients; CON, control subjects.

associations when we set the genotype relative risk at each value as shown in Table 3.

Discussion

Two-stage association analyses with quite high power revealed no association between chromosome 5 $GABA_A$ cluster and Japanese schizophrenia.

We adopted a reasonable strategy for association analyses (van den Oord and Neale 2004) that is more powerful than ones with randomly selected SNPs (Kamatani et al 2004). Furthermore, we carefully treated the inflation of type I error rate due to multiple testing: the independent panel of samples and the adjustment considering dependence between SNPs (by SNPSpD). Our results indicate the clear importance of correcting the inflation of the type I error rate in genetic association analysis when increasing the number of markers examined. A recent simulation showed that increasing the sample size is more powerful than continuously increasing the number of SNPs (Huang et al 2003); thus, we did not genotype additional SNPs in the vicinity of SNP20 and SNP24. Of course, the false positive results in the first-set analysis might be derived from population stratification. Genomic control may be required for conclusive results; however, we speculate that this was not the case, because Japanese population is believed to be homogeneous.

We found a unique LD structure of GABRA1 in initial evaluation of LD, as follows: although the LD matrix between the SNPs of GABRA1 located farthest from each other (SNP19 and SNP30) had strong LD, some pairwise LD matrices within GABRA1 had low D' values (for example, among SNP20/21 and SNP28/29). This structure is unlikely to be in accordance with the genetic model in which LD blocks are dictated by recombination hotspot (Goldstein 2001). Thus, we applied this LD pattern of GABRA1 to stricter criteria of LD (Wall and Pritchard 2003a), although no concept fit this LD pattern. In such a case as this, it is necessary to consider the possibility of genotyping error. At first, we checked the D' values among these markers with first-set samples (288 cases and 288 control subjects). Also, same trends were obtained (D' between SNP20 and SNP29 = .23 [cases], .24 [control]; D' between SNP21 and SNP29 = .16 [cases], .33 [control]). Next, we re-checked the genotyping of "signal" htSNPs, SNP20 and SNP24, with a different method (initial genotyping was by PCR-RFLP), the direct sequencing method (done for 96 cases and 96 control subjects, not all subjects). The results were identical to the initial results. Hence, we speculate that it was unlikely that genotyping error had occurred, and that this unique LD pattern was not related to false positive results in first-set analysis.

We could not replicate a previously reported positive association of SNPs (SNP2-SNP6 and SNP8 in Table 1) in GABRB2 in Han Chinese (Lo et al 2004). To avoid redundant results, in this study we did not genotype all positive SNPs, because our sample showed tight LD patterns different from those of the Han Chinese sample and our "htSNP" might represent these positive SNPs. Our data support a difference of LD structure in study populations (Wall and Pritchard 2003b), and for confirmation we consulted the HapMap data (http://www.hapmap.org/; HapMap public release #15; accessed February 18, 2005) around this region in Japanese and in Chinese populations. Unfortunately, these data did not contain the positive SNPs; however, the regions included in these SNPs (between rs252942 and rs967771) were strong LD (D'=1) in both populations. We assume that LD patterns among these SNPs in Chinese might also be complex, whereas the LD patterns of Japanese were similar to those of HapMap data (when we excluded SNP7, which were not analyzed by Lo et al [2004]).

The most recently reported association study between GABRA1 and Caucasian schizophrenia showed a significant association (Petryshen et al 2004). Our results could not support Petryshen's findings, but further replication study will be required with other samples from different populations.

A few points of caution should be noted in interpreting our results. First, the lack of association may be due to biased samples, especially unmatched age samples and ascertainment bias of control subjects. Second, we must consider the interaction of other candidates related to this $GABA_A$ receptor gene cluster, such as other $GABA_A$ receptor genes on 4p and 15q, glutamic acid decarboxylase and others.

In conclusion, these results, obtained with one of the optimal strategies for genetic association analysis, indicate that this gene cluster may not play a major role in Japanese schizophrenia. They also raise an alert with regard to preliminary genetic association analysis with a small sample size, and indicate that a replication analysis using large samples is required for reliable results that avoid type I and type II errors.

We thank Ms. M. Miyata, Ms. S. Nakaguchi for their technical support. This work was supported in part by research grants from the Ministry of Education, Culture, Sports, Science, and Technology, and the Ministry of Health, Labor, and Welfare.

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International Journal of Neuropsychopharmacology (2005), 8, 1-5. Copyright © 2005 CINP
doi:10.1017/S1461145705005481

BRIEF REPORT

Positive association of AKT1 haplotype to Japanese methamphetamine use disorder

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Abstract

Recent evidence suggests that the AKT1-GSK3β signalling cascade partially mediates dopamine-dependent behaviours. In relation to the pathophysiology of schizophrenia or methamphetamine (Meth) use disorder, AKT1 is a good candidate gene for such conditions. For schizophrenia, positive associations of SNPs and AKT1 haplotypes were reported in US and Japanese samples. To evaluate the association between AKT1 and Meth-use disorder, we conducted a case-control study of Japanese samples (182 patients and 437 controls). A positive association between a SNP and haplotypes was found, and the 'signal' SNP was the same SNP found to be associated with US schizophrenia, but not with Japanese schizophrenia. Our results indicate that AKT1 may play a possible role in the development of Meth-use disorder. Further investigation of these associations, together with evidence from previous animal studies, may open the way to elucidation of the pathophysiology of this condition.

Received 20 October 2004; Reviewed 21 December 2004; Revised 11 January 2005; Accepted 19 January 2005

Key words: Dopamine-dependent behaviours, linkage disequilibrium, substance-related disorders.

Introduction

The pathophysiology of methamphetamine (Meth) use disorder has not been well established, however, one of the most likely mechanisms is abnormality of the dopamine (DA) neurotransmission system. The pharmacological profile of Meth shows that the target site of Meth is the DA transporter (DAT). Also the mesolimbic DA system has an important function in reinforcement and reward mechanisms (Spanagel and Weiss, 1999).

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Family and twin studies suggested that the genetic contribution is important in that it may predispose certain people to this disorder (Tsuang et al., 1996, 1998). Recent studies have suggested that V-akt murine thymoma viral oncogene homologue 1 (AKT1) is a good candidate for a Meth-use disorder susceptibility gene, for the following reasons. (1) An animal study of DAT knock-out (KO) mice and wild-type mice, treated with lithium salts and amphetamine, showed that the AKT1–glycogen synthase kinase 3β (GSK3β) signalling cascade partially mediated DA-dependent behaviours (Beaulieu et al., 2004). (2) AKT1 KO mice treated with amphetamine showed a reduction in prepulse inhibition (PPI) (Emamian et al., 2004). PPI disturbances are known to be present in schizophrenia, which might also be related to

abnormalities in the DA system. AKT1 haplotypes were shown to have a significant association with schizophrenia in a transmission disequilibrium test (TDT) study (Emamian et al., 2004) and in a previous Japanese case-control replication study by the authors (Ikeda et al., 2004), although no association was found in another Japanese replication study (Ohtsuki et al., 2004).

Here we conducted a case-control study of Japanese Meth-use disorder samples using the single nucleotide polymorphisms (SNPs) of our previous study to evaluate the association of AKT1 with Meth-use disorder.

Methods

A total of 182 patients with Japanese Meth-use disorder [146 male, 36 female; mean age ± standard deviation (S.D.), 36.7 ± 12.0 yr] and 437 controls (209 male, 228 female; 34.3 ± 13.6 yr) were analysed. The number of patients with Meth-use disorder comprised of 168 Meth-dependent subjects, and 14 Meth-abuse subjects. Among the subjects with Meth-use disorder, 153 subjects (127 males, 26 females) have a comorbid diagnosis of Meth-induced psychosis, three of anorexia nervosa, one of obsessive–compulsive disorder, and one of major depressive disorder. And 120 subjects with Meth-use disorder have abuse or dependence on drugs other than Meth. Subjects with Meth-use disorder were excluded if they had a comorbid diagnosis of any psychotic disorder other than Meth-induced psychosis. 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. All healthy controls were also psychiatrically screened based on unstructured interviews. More details about the characterization of these subjects have been published elsewhere (Suzuki et al., 2003; Ujike et al., 2003). After description of the study, written informed consent was obtained from each subject. This study was approved by the Ethics Committee at each participating institute of the Japanese Genetics Initiative for Drug Abuse (JGIDA).

For SNP genotyping, polymerase chain reaction (PCR) amplification, restriction fragment length polymorphism (RFLP) assays were developed; *Bsa*I for SNP1 (rs3803300), *Xcm*I for SNP2 (rs1130214), *Hae*III for SNP3 (rs3730358), *Hpy*CH4IV for SNP4 (rs2498799), *Pst*I for SNP5 (rs2494732), and *Bsi*HKAI for SNPA (rs2498804). A detailed description may be found in a previous report (Ikeda et al., 2004) and information about primer sequences and PCR–RFLP conditions are available on request.

Hardy–Weinberg equilibrium (HWE) was evaluated by conventional χ^2 test (SPSS 10.0J, SPSS Japan Inc., Tokyo, Japan). For marker-trait association analyses, we constructed multi-SNP haplotype systems (Emamian et al., 2004) to evaluate the association through permutation p values in sliding window fashion and global p values respectively. In total sample association analysis (not in explorative association analysis), we emphasize the permutation p values over the respective global p values because the permutation procedure gives a significance corrected for the multiple haplotypes and markers tested. Furthermore, we corrected these permutation p values by Bonferroni correction to obtain more robust results. A more detailed description is given in our previous paper (Ikeda et al., 2004).

We also include an explorative analysis for gender effects, because of the following reasons. (1) Aetiological study suggests that the genetic contribution of substance-related disorder is differentially heritable by gender (Jang et al., 1997). (2) Our samples were unmatched gender ratios of Meth-use disorder (36 female, 146 male).

Results

Genotype frequencies of all SNPs were in HWE. Positive permutation p values of 4- and 5-marker sliding window fashion ($p = 0.0083$ and 0.023 respectively) and global p value of 6-marker combinations ($p = 0.017$) were obtained. One of the 4-marker sliding window fashion p values remained significant ($p = 0.0498$) even after Bonferroni correction was performed six times (once for single marker permutation and five times for haplotype combinations). In the single marker association analysis (i.e. a conventional allele-wise association analysis), only SNP3 was associated with Meth-use disorder ($p = 0.019$) (Table 1).

Individual haplotypic analyses from the positive global 4-marker p values are shown in Table 2. The haplotype with the most significant association was more frequent in controls than in cases (SNP1-2-3-4, G-G-C-G, $p = 0.0032$).

Explorative analysis of gender effects is shown in Table 3. In female samples, eight of 21 global p values showed significance. In these significant p values, SNP3, which was associated with total Meth-use disorder, showed strong association ($p = 0.0011$). On the other hand, the positive global p values in male samples tended to be similar to those in total samples (positive global p values: SNP1-2-3-4 = 0.036, SNP1-2-3-4-5-A = 0.042), however, SNP3 was not associated with male Meth-use disorder ($p = 0.11$).