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No association between monoamine oxidase A promoter polymorphism and personality traits in Japanese females

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Abstract

Monoamine oxidase A (MAO-A) is an enzyme involved in the metabolism of monoamine neurotransmitters such as dopamine, serotonin, and noradrenaline in the brain. Previous studies have demonstrated a significant association between MAO-A gene polymorphism and personality traits in males. The purpose of the present study was to examine this association in females. The subjects were 219 healthy Japanese females. We genotyped a variable number of tandem repeats located upstream of the MAO-A gene. Personality traits were assessed using the Temperament and Character Inventory (TCI). There was no association between any personality trait and MAO-A genotype. The present results do not support the hypothesis that MAO-A gene polymorphism is related to certain personality traits in females.

Keywords: Monoamine oxidase A; Personality; TCI; Polymorphism; Gene

Monoamine oxidase (MAO) is a mitochondrial enzyme that catalyzes the degradation of several different biological amines, and includes two distinguishable forms: MAO-A and MAO-B. The former is present in the catecholaminergic neurons of the brain, and has a preference for noradrenaline, adrenaline, and serotonin as substrates, whereas the latter is present in the serotonergic neurons of the brain, and prefers β-phenylethylamine as a substrate. Both forms can oxidize dopamine, tyramine, and tryptamine [13].

According to Cloninger's model, human personality consists of seven dimensions including three temperament dimensions and four character dimensions, and on the basis of this model Cloninger developed the Temperament and Character Inventory (TCI), a questionnaire for assessing personality traits [2]. The three temperament dimensions, which include Novelty Seeking, Harm Avoidance, Reward Dependence, and Persistence, have been assumed to be related to monoamine neurotransmitters: Novelty Seeking with dopaminergic activities, Harm Avoidance with serotonergic activities, and Reward Dependence with noradrenergic activities (Table 1). Since MAO is involved in the metabolism of these monoamine neurotransmitters, this enzyme in the brain might be closely related to human personality measured by the TCI.

Additionally, Brunner et al. [1] reported the Brunner syndrome, caused by a deficiency in MAO-A, which has features of borderline mental retardation and repetitive violent behavior. The restriction of dopaminergic activities resulting from MAO-A deficiency is considered to contribute to the aggressive behavior. Following this, there was an increased number of studies that investigated the relationship between the MAO-A gene and behavioral traits.

MAO-A is encoded by a single gene located on bands Xp11.23. A polymorphism located 1.2 kb upstream of the MAO-A coding sequences has been shown to affect the

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Table 1
Comparison of the Temperament and Character Inventory (TCI) subitem score among 3/3 vs. 3/4 and 4/4 repeats of MAOA gene polymorphism

TCI subitem	N[a]		Score[b]		P value
	3 × 3	3 × 4 and 4 × 4	3 × 3	3 × 4 and 4 × 4	
Novelty Seeking	79	129	22.3 ± 6.5	21.3 ± 5.6	0.23
Harm Avoidance	74	119	17.8 ± 6.5	17.9 ± 6.6	0.93
Reward Dependence	80	133	16.6 ± 3.6	17.4 ± 3.4	0.13
Persistence	79	133	4.8 ± 2.0	4.8 ± 2.1	0.90
Self-directedness	79	125	26.1 ± 6.2	26.6 ± 7.2	0.61
Cooperativeness	71	117	29.5 ± 5.5	30.3 ± 5.3	0.34
Self-transcendence	78	124	14.9 ± 5.8	14.8 ± 5.2	0.84

[a] Of a total of 219 subjects examined, 5 were excluded because of not submitting the TCI answer sheet. Since several blanks are found on each dimension in the TCI answer sheet of the remaining 214 subjects, the total number of subjects is different depending on each dimension.
[b] Values are expressed as mean ± standard deviation.

transcriptional activity of the MAO-A gene promoter. This mutation consists of a 30-bp repeated sequence present in 3, 3.5, 4, or 5 copies, each associated with different transcriptional efficiency; alleles with 3.5 or 4 copies of the repeat sequence are transcribed 2–10 times more efficiently than those with 3 copies of the repeat [3,14].

An association between this gene polymorphism (30-bp VNTR) and behavioral traits has been found in several studies. For example, Manuck et al. [11,12] reported that male subjects with 3.5 and 4 repeats scored significantly higher on scales of aggression than those with 3 and 5 repeats. On the other hand, Eley et al. [4] reported that males with high Neuroticism scores were significantly more likely to have 3.5 or more repeats than low scorers, while Garpenstrand et al. [5] and Jorm et al. [7] reported no such significant association. So far, the association between MAO-A gene polymorphism and behavioral traits in females has not been investigated.

The aim of the present study was to investigate the possible relationship between genotypes of MAO-A promoter polymorphism and behavioral traits in Japanese females.

This study was carried out after obtaining approval from the Ethics Committee of Nagoya University School of Medicine and Keio University School of Medicine. To minimize the effects of confounding variables such as race, age, and gender, the subjects enrolled were all Japanese female students in their first year at a nursing school ($n = 219$ females). Their mean age was 20 years (range: 18–32 years). After giving the subjects a full description of the study, written informed consent to participate was obtained from each of them.

The subjects were asked to complete the 240 items of the Japanese version of the TCI [2], whose reliability and validity had been established by Kijima et al. [8].

DNA was extracted from peripheral lymphocytes according to the standard method. Genotyping was carried out according to the standard protocol, with a slight modification from the published methods [4,11]. Each target segment was amplified by the polymerase chain reaction method and subjected to electrophoresis.

For statistical analysis, the 'SPSS for Windows ver11.0' (SPSS Japan Inc., Tokyo, Japan) software package was used. Genotype deviation from the Hardy–Weinberg equilibrium was evaluated by chi-squared test. Since MAO-A promoter activity is known to be decreased in subjects with 3/3 genotypes, and to be increased in subjects with 3/4 and 4/4 genotypes [3,14], the TCI scores were compared between 3/3 genotypes and others (3/4 and 4/4). The mean scores for the seven factors (Novelty Seeking, Harm Avoidance, Reward Dependence, Persistence, Self-directedness, Cooperativeness, Self-transcendence) of the TCI were compared among the genotypes (3/3 versus 3/4 and 4/4) using Student's t-test. The result was defined as being significant at $P < 0.05$.

The numbers of the genotypes 3/3, 3/4, and 4/4 were 83, 108, and 28, respectively. The 3.5 repeat allele was not detected at all in our samples. The genotype distribution was not significantly different from that expected according to the Hardy–Weinberg equilibrium. There were no significant differences in the mean scores for the seven TCI factors between the genotypes (3/3 and 3/4, 4/4).

The purpose of the present study was to examine the association between MAO-A gene polymorphism and personality traits. We detected no significant association between MAO-A genotype and TCI score, indicating that MAO-A gene promoter polymorphism is unlikely to have affected the personality traits in our sample. These results are consistent with other previous reports [5,7]. MAO-A is a crucial enzyme in the central nervous system, and Sabol et al. [14] reported that 3 repeats reflected low MAO-A activity, whereas 3.5 and 4 repeats reflected high MAO-A activity in vitro. However, on the basis of the present results, it seems unlikely that the MAO-A function reflected by this polymorphism is associated with the formation of personality traits.

Although the 3.5 repeat allele was not detected among our samples, this finding is in accordance with previous studies carried out in Japan. In fact, the distribution of the allele frequency in these studies was very similar to that observed in the present study [6,9].

The differences between our results and the previous studies showing a positive association between MAO-A and personality traits [4,11,12] may have been derived from differences in the demographic background of the

subjects and of the questionnaire used for personality evaluation.

It can be considered that the present study had a few limitations. First, our subjects were a fairly specifically defined population with only a narrow range of demographic factors, since they were all female students attending a nursing school, and thus their personality traits may have shown a similar trend. Second, there was a possibility of type II error in detecting significant differences, due to an insufficient sample size. Based on the results of power analysis [10], if the type I error is set at 0.05 and the power at 0.80, at least 285 samples are theoretically required to detect a significant difference of Reward Dependence, which showed the smallest *P* value observed in the present study.

Considering the relationship between MAO-A gene deficiency and violent personality, the possibility of some involvement of MAO-A polymorphism in personality traits cannot be ruled out. In addition, since other mechanisms regulating neurotransmitters such as COMT, MAO-B, and TPH2 may also be involved in compensation for altered MAO-A activity, relationships with these polymorphisms may also need to be considered. Further research with additional genotyping of other dopamine-related gene polymorphisms is needed using additional subjects with various demographic characteristics, in order to demonstrate more clearly any potential role of MAO-A polymorphism in the formation of personality traits.

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Positive Association of the Serotonin 5-HT$_7$ Receptor Gene with Schizophrenia in a Japanese Population

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Several lines of evidence suggest that abnormalities in the serotonin system may be related to the pathophysiology of schizophrenia. The 5-HT$_7$ receptor is considered to be a possible schizophrenia-susceptibility factor, based on findings from binding, animal, postmortem, and genomewide linkage studies. In this study, we conducted linkage disequilibrium (LD) mapping of the human 5-HT$_7$ receptor gene (HTR7) and selected four 'haplotype-tagging (ht) SNPs'. Using these four htSNPs, we then conducted an LD case-control association analysis in 383 Japanese schizophrenia patients and 351 controls. Two htSNPs (SNP2 and SNP5) and haplotypes were found to be associated with schizophrenia. A promoter SNP (SNP2) was further assessed in a dual-luciferase reporter assay, but it was not found to have any functional relevance. Although we failed to find an actual susceptibility variant that could modify the function of HTR7, our results support the supposition that HTR7 is a susceptibility gene for schizophrenia in this ethnic group.
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Keywords: schizophrenia; 5-HT$_7$ receptors; linkage disequilibrium; htSNP; haplotype

INTRODUCTION

The human 5-hydroxytryptamine 7 (5-HT$_7$) serotonin receptor is a G-protein-coupled receptor (Hoyer *et al*, 2002). These receptors are expressed in discrete areas of the brain as well as in both vascular and gastrointestinal smooth muscle (Bard *et al*, 1993; Lovenberg *et al*, 1993), and the recent availability of selective antagonists and knockout mice strains has increased our knowledge about their functional roles. The 5-HT$_7$ receptors are now known to have an important role in modulating hippocampal neuronal functions such as learning and memory, disturbances in which are thought to be fundamental in schizophrenia (Hedlund and Sutcliffe, 2004; Thomas and Hagan, 2004). 5-HT$_7$ receptor knockout mice have also exhibited a specific impairment in contextual fear conditioning, which is associated with hippocampus-dependent learning, and reduced ability for long-term potentiation in the CA1 region of the hippocampus (Roberts *et al*, 2004).

Other *in vivo* and *in vitro* studies suggest that 5-HT$_7$ receptors can mediate the effects of 5-HT on hippocampus glucocorticoid receptor expression (Weaver *et al*, 2001; Laplante *et al*, 2002; Beique *et al*, 2004). Glucocorticoid has been tentatively associated with neurotoxicity, and considering the neurodevelopmental hypothesis of schizophrenia might have some role in this disease (Cotter and Pariante, 2002).

Neuropsychopharmacologically, 5-HT$_7$ receptors show high affinity for a number of antidepressants and typical and atypical antipsychotics. For example, clozapine, which is a classical atypical antipsychotic drug and which has been shown to be effective in treating schizophrenia (Safferman *et al*, 1991; Kane *et al*, 2001), exhibits moderate potency as a 5-HT$_7$ receptor antagonist. This finding suggests that an interaction between and/or overactivity of receptor systems may be involved in the pathophysiology of schizophrenia, although the pharmacological basis of the unique actions of clozapine is not well understood. Other lines of evidence also support the association of 5-HT$_7$ receptors to schizophrenia; A postmortem study showed decreased expression of 5-HT$_7$ receptors in the dorsolateral prefrontal cortex of schizophrenics (East *et al*, 2002). Furthermore, genomewide linkage studies of schizophrenia have shown a linkage in 10q22 (Mowry *et al*, 2000; Fallin *et al*, 2003) (OMIM: SCZD11,%608078) close to the location of the human 5-HT$_7$ receptor gene (HTR7: 10q21–24).

Here, we evaluate whether HTR7 is associated with schizophrenia in a sample including 383 unrelated Japanese schizophrenia patients and 351 unrelated controls. A common hypothesis about allelic architecture proposes that

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the greatest genetic risk for common, complex diseases including schizophrenia is from disease loci that have one common variant (common disease–common variants hypothesis: CD–CV hypothesis) (Chakravarti, 1999). In the light of this CD–CV hypothesis, association analysis using linkage disequilibrium (LD) mapping would seem to be for a reasonable approach to narrow down the number of potential susceptibility genes or variants for schizophrenia. We therefore performed (1) LD mapping in HTR7 and selection of 'haplotype-tagging (ht) SNPs', (2) an association analysis using 'htSNPs', and (3) a systematic mutation search to detect actual susceptibility variants capable of modifying the function of HTR7.

MATERIALS AND METHODS

Subjects

A total of 383 patients with schizophrenia (200 male and 183 female; mean age ± standard deviation (SD), 42.6 ± 14.5 years) participated in the present study. The subjects for the mutation search were 48 schizophrenic patients who were also among the 383 patients in the association analysis (27 male and 21 female; mean age ± SD, 39.5 ± 14.7 years). A total of 351 healthy volunteers (193 male and 158 female; mean age ± SD, 33.5 ± 13.1 years) were recruited as control subjects. The subjects for initial 'LD mapping' were 96 controls who were also subjects in the association analysis.

The characterization details and psychiatric assessment of these subjects were identical to those published elsewhere (Suzuki *et al*, 2003; Ikeda *et al*, 2005). 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 controls were also psychiatrically screened based on unstructured interviews. All subjects were unrelated to each other and ethnically Japanese, and were individually matched for gender and geographical origin.

After the study had been described, 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 Inclusion for LD Mapping

We included all SNPs from the dbSNP database (URL; http://www.ncbi.nlm.nih.gov/SNP/) and Celera Discovery System database (URL; http://www.celeradiscoverysystem.com/) for LD mapping (Table 1 and Figure 1).

Selection of 'htSNPs'

'LD blocks' were first determined using criteria based on 95% confidential bounds on D' values (Gabriel *et al*, 2002), with Haploview version 2.05 software (Barrett *et al*, 2004). 'htSNPs' were then selected within each 'LD block' for 90% haplotype coverage using SNPtagger software (Ke and Cardon, 2003). This strategy for association analyses after initial LD mapping and 'htSNPs' selection is considered to be reasonable on the basis of descriptions given in other papers (Kamatani *et al*, 2004; van den Oord and Neale, 2004).

SNP Genotyping

Genomic DNA was extracted from peripheral blood of all subjects. For rapid genotyping of SNPs, we used TaqMan assays (SNP1, SNP3, SNP4, SNP5, and SNP7), polymerase chain reaction (PCR)-restriction fragment length polymorphism (RFLP) assays (SNP6), primer extension with denaturing high performance liquid chromatography (dHPLC) (SNP2) and direct sequencing (SNP8) (Table 1). TaqMan probes and Universal PCR Master Mix were from Applied Biosystems (CA). A 5-μl total reaction volume was used and, after PCR, the allelic specific fluorescence was measured on an ABI PRISM 7900 Sequence Detector System (Applied Biosystems, CA). Methods for RFLP assays, primer extension, and direct sequencing were described in greater detail previously (Suzuki *et al*, 2003).

Dual-Luciferase Reporter Assay

Fragments of 262 bp including SNP2 were PCR amplified. Genomic DNAs with identified genotypes were used as templates, and PCR products of both genotypes were cloned into pGL3-promoter Vector (Promega, WI). These Vectors with both alleles and the *Renilla* luciferase vector, phRL-TK vector, were transiently transfected into Chinese hamster ovary (CHO) cells using Lipofectamine 2000™ (Invitrogen, CA). After 48 h, cell extracts were prepared and assayed for firefly luciferase activity (LA_F) and *Renilla* luciferase activity (LA_R) as described by the manufacturer (PikkaGene Dual SeaPansy™ Luminescence Kit, Toyo Ink, Japan) on a Fluoroskan Ascent FL (Thermo Labsystems, Finland). All experiments were repeated at least three times. To correct for transfection efficiency, the relative luciferase activity (RLA) was calculated as: $RLA = LA_F/LA_R$. The RLA of mutants (RLAmt) was shown by % ratio of the RLA of wildtype (RLAwt). Means and standard errors were calculated and an unpaired two-tailed t-test was performed using the software package SPSS 10.0J (SPSS Japan Inc.,

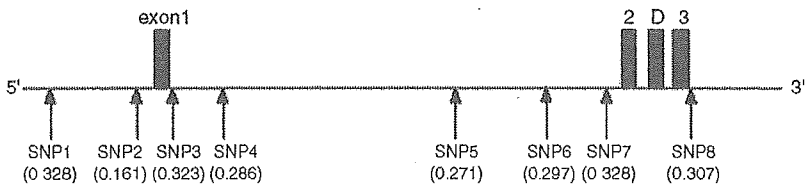

Figure 1 Genomic structure of HTR7 with SNPs used in LD mapping. Exon D is an alternative splice exon. Numbers in parentheses represent minor allele frequencies (MAFs) of 96 controls.

Japan). Further details about primer pairs and culture conditions for the cells are available on request.

Mutation Search

We performed dHPLC analysis, details of which can be seen in a previous paper (Suzuki *et al*, 2003). Primer pairs were designed using information from the GenBank sequence (accession number: NM-030059.11) and Erdman's paper (Erdmann *et al*, 1996), as well as from 14 amplified regions that covered all the exons, alternative splice exon (exon D), and introns including the branch sites and 1000 bp upstream from initial exon of HTR7.

Statistical Analysis

Genotype deviation from the Hardy–Weinberg equilibrium (HWE) was evaluated by χ^2 test (SAS/genetics, release 8.2, SAS Institute Japan Inc., Tokyo, Japan).

Marker–trait association analysis was performed with the COCAPHASE 2.403 program (Dudbridge, 2003). This program performs log-likelihood ratio tests under a log-linear model for global *P*-value. To estimate haplotype frequencies, an expectation–maximization (EM) algorithm was used (Zhao *et al*, 2000). Rare haplotypes found in less than 3% of both cases and controls were excluded from association analyses due to the limitation of the EM algorithm. For haplotypic analyses, we calculated global *P*-values in multi-SNP haplotype systems. In both haplotypic and single marker allelewise analyses, we performed the permutation procedure that is a tool in the COCAPHASE program in a 1- to 3-marker sliding window fashion. We emphasized the permutation *P*-values rather than individual global *P*-values, because this permutation method is considered to be appropriate for these analyses (Dudbridge, 2003) (ie Bonferroni correction for multiple testing is considered to be too conservative to apply to genetic association analyses (Nyholt, 2001)). Ten thousand permutations were performed in each permutation procedure, and ORs (ORs) of risk alleles or haplotypes were calculated when the most frequent alleles or haplotypes had ORs of 1. To detect risk haplotypes, we performed the individual procedures in this program. The significance level for all statistical tests was 0.05.

RESULTS

LD Mapping

We genotyped eight SNPs for 96 controls. After testing for deviation from HWE (all SNPs were in HWE), we evaluated pairwise LD matrices between each SNP, and selected 'htSNPs' from LD blocks. Consequently, we determined three 'LD blocks' and four 'htSNPs' (SNP1, SNP2, SNP5, and SNP7) (Table 2).

Association Analysis

We expanded genotyping of these four 'htSNPs' for all the schizophrenia subjects and the remaining 255 controls. In this step, genotype distributions were again in HWE.

Table 1 Summary of SNPs in HTR7

SNP ID	Distance to next SNP (bp)	Methods	Primer sequences for PCR		Primer sequence for primer extension	Restriction enzyme
			Forward	Reverse		
SNP1 (C>T) rs10509613 (C206914)	—	TaqMan				
SNP2 (G>A) rs3808932	34915	Primer extension	CTTCCCATAAGCGGTCAAAA	GTCCTTGCCGCATGATAGAT	GAGACGTTTTTTTGGTTGTT	
SNP3 (T>C) rs7919103 (C368389)	3019	TaqMan				
SNP4 (G>A) rs2185706 (C1756841)	12040	TaqMan				
SNP5 (G>A) rs12412496 (C416286)	56980	TaqMan				*Sna*I
SNP6 (A>C) rs2420367 (C1756716)	19982	PCR-RFLP	TTTTCCATCCCACTTTCAGC	ACAGGCAGAAAACAGCGAAC		
SNP7 (C>T) rs4933194 (C375195)	14816	TaqMan				
SNP8 (A>G) rs1045949	10643	Direct sequence	AGGGCACTGTCCTTTCAGAC	ATGTCTGCCCTTCAGCCATT		

Table 2 Pairwise Linkage Disequilibrium (LD) Matrices and 'LD Blocks' in HTR7

		BLOCK1			BLOCK2		BLOCK3			
Block[a]	SNPID	SNP1	SNP2	SNP3	SNP4	SNP5	SNP6	SNP7	SNP8	MAF[b] (%)
BLOCK1	SNP1		1.0 (0.56–1.0)	1.0 (0.95–1.0)	0.71 (0.33–0.88)	0.77 (0.37–0.92)	0.83 (0.71–0.91)	0.85 (0.76–0.92)	0.78 (0.66–0.87)	32.8
	SNP2	0.09		1.0 (0.54–1.0)	1.0 (0.86–1.0)	0.90 (0.71–0.97)	0.77 (0.21–0.93)	0.82 (0.32–0.95)	0.76 (0.20–0.93)	16.1
	SNP3	0.98	0.09		0.79 (0.42–0.93)	0.88 (0.48–0.97)	0.83 (0.72–0.91)	0.88 (0.78–0.94)	0.79 (0.66–0.87)	32.3
BLOCK2	SNP4	0.10	0.48	0.12		1.0 (0.93–1.0)	0.78 (0.40–0.92)	0.82 (0.50–0.94)	0.79 (0.41–0.92)	28.6
	SNP5	0.11	0.42	0.14	0.93		0.87 (0.47–0.97)	0.90 (0.57–0.98)	0.88 (0.48–0.97)	27.1
BLOCK3	SNP6	0.60	0.05	0.62	0.10	0.12		1.0 (0.93–1.0)	0.95 (0.87–0.99)	29.7
	SNP7	0.73	0.06	0.75	0.13	0.15	0.86		0.95 (0.86–0.99)	32.8
	SNP8	0.56	0.05	0.57	0.11	0.13	0.86	0.82		30.7

[a]Each BLOCK was determined by HAPLOVIEW.
[b]MAF = minor allele frequencies of 96 controls in LD mapping.
Numbers of upper diagonals are D′ and parenthetical numbers are 95% confidential intervals of D′. Numbers of lower diagonals are r^2.
Boxes in gray represent 'ht SNPs'.

Table 3 Association Analyses of Four 'ht SNPs' in HTR7

	Multi-SNP haplotype systems[a]				Genotypic distribution[b]							Allelic distribution MAF (%)	
					M/M		M/m		m/m				
SNPID	1SNP	2SNP	3SNP	4SNP	SCZ	CON	SCZ	CON	SCZ	CON	P-values (genotype)	SCZ	CON
SNP1	0.347				177	147	156	157	50	47	0.477	33.4	35.8
		0.0274											
SNP2	**0.00571**		**0.0137**		231	240	124	98	28	13	**0.0257**	23.5	17.7
		0.00584		**0.0398**									
SNP5	**0.00504**		**0.0280**		162	175	168	149	53	27	**0.0128**	35.8	28.9
		0.0313											
SNP7	0.664				168	147	159	152	56	52	0.856	35.4	36.5
Permutation P-value[c]	***0.0197***	***0.0386***	***0.0424***										

[a]P-values were calculated by log-likelihood ratio test (1SNP; allelewise association, SNP2–4; global haplotypic association). Bold numbers represent significant P-values. Bold italics numbers represent significant P-values of permutation test.
[b]M = major allele; m = minor allele; SCZ = schizophrenia; CON = control; MAF = minor allele frequency.
[c]An implement in the program COCAPHASE.

Almost all global *P*-values except 1-marker global *P*-values of SNP1 and SNP7 were associated with schizophrenia. After adjustment by permutation procedure, we found a significant association of all permutation *P*-values with schizophrenia (Table 3). An assessment of the components of these significant associations indicated SNP2 and SNP5 as possible key components for schizophrenia ('A' allele of SNP2, case = 23.5%, control = 17.7%, and *P*-value = 0.00571; 'A' allele of SNP5, case = 35.8%, control = 28.9%, and *P*-value = 0.00504; Table 3). The ORs for the significant risk genotypes are modest; OR = 1.43 (95% CI: 1.11–1.85) for SNP2, OR = 1.37 (95% CI: 1.10–1.71) for SNP5.

To detect the risk haplotypes, individual haplotypic analyses were performed from positive global *P*-values (Table 4). The most significant haplotype overtransmitted in schizophrenia was a combination of SNP2 and SNP5 ('A–A' haplotype, case = 23%, control = 16%, and *P*-value = 0.0015); however, the estimated haplotype frequencies of each risk haplotype were nearly equal to the minor allele frequency (MAF) of SNP2 (case = 23.5%, control = 17.7%).

Dual-Luciferase Reporter Assay of SNP2

Taken together with the results of the LD mapping (SNP2 was in strong LD with SNP5), we hypothesized that SNP2, a possible promoter SNP (533 bp from initial exon), may be a susceptibility SNP for schizophrenia. To confirm the functional relevance of SNP2, we developed a dual-luciferase reporter assay. However, the results from this assay did not provide evidence that SNP2 modifies transcriptional activity of HTR7 (*P*-value = 0.782; RLAwt = 100 ± 58.2, RLAmt = 86.2 ± 45.2).

Table 4 Individual Haplotypic Analyses from Positive Permutation Analyses of Schizophrenia

Combination of SNPs	Marker haplotype	Frequency SCZ	Frequency CON	P-values	OR
SNP1-2	C–G	0.43	0.47	NS	1
	C–A	0.23	0.18	0.0068	1.42
	T–G	0.33	0.36	NS	
SNP2-5	G–G	0.64	0.70	0.013	1
	G–A	0.13	0.13	NS	
	A–A	0.23	0.16	0.0015	1.54
SNP5-7	G–C	0.32	0.37	0.043	1
	G–T	0.32	0.34	NS	
	A–C	0.33	0.27	0.010	1.41
SNP1-2-5[a]	C–G–G	0.33	0.37	NS	1
	C–A–A	0.23	0.16	0.0015	1.59
SNP2-5-7[a]	G–G–C	0.32	0.35	NS	1
	A–A–T	0.21	0.15	0.0035	1.59
SNP1-2-5-7[a]	C–G–G–C	0.30	0.34	NS	1
	C–A–A–C	0.21	0.15	0.0041	1.57

SCZ = schizophrenia; CON = control; NS = not significant; OR = odds ratio.
[a]Only reference haplotypes and positive haplotypes are shown.

Mutation Search

To detect the actual susceptibility variants, we performed a systematic mutation search in all exons and introns including branch sites and 1000 bp upstream from the initial exon. However, we could not find any functional mutations in these regions.

DISCUSSION

In this study, two htSNPs (a possible promoter SNP (SNP2) and an intronic SNP (SNP5)) and haplotypes showed an association with schizophrenia in a Japanese population. However, the functional relevance of SNP2 could not be confirmed with the dual-luciferase reporter assay.

The method of using 'htSNPs' in LD association analyses is more sensitive and powerful than that of using randomly selected SNPs. Here, selection of 'htSNPs' resulted in the fitting of another criteria, 'LD-selected htSNPs', at a relatively stringent r^2 threshold ($r^2 > 0.8$) (Carlson *et al*, 2004). This method of 'LD-selected SNPs' reflects the evolutionary relationships of haplotypes. Therefore, our 'htSNPs' could identify disease associations with either specific haplotypes or with clades of related haplotypes (Carlson *et al*, 2004). Given this LD pattern of HTR7, the actual susceptibility variants may exist anywhere in HTR7.

Although our sample size in this mutation search was sufficient to detect common variants with more than 5% MAF at 95% power (Collins and Schwartz, 2002), we failed to find the actual susceptibility variants through a systematic mutation search. Since the actual promoter region in HTR7 has not been determined, the regions targeted in our mutation search might not have been adequate. In addition, we did not search the conserved noncoding sequence that plays a role in gene regulation. Further investigation will be required for conclusive results.

An additional point deserves attention in interpreting the results: The positive association with schizophrenia could be due to type I error, possibly because of population stratification or unmatched age samples. Replication study with genomic control or a family-based population will be required.

In conclusion, we found the first, and a significant, association of SNPs in HTR7 with Japanese schizophrenia patients after consideration of problems in statistical genetics. These results support the supposition that HTR7 is a schizophrenia-susceptibility gene.

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Behavioral/Systems/Cognitive

A Variant C178T in the Regulatory Region of the Serotonin Receptor Gene *HTR3A* Modulates Neural Activation in the Human Amygdala

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Converging evidence in neurophysiological and neuroimaging studies has suggested that the limbic and prefrontal systems play important roles in emotion and cognition. These structures are activated when we see a human face, assuming that we automatically evaluate the biological significance of the stimuli. The serotonin (5-HT) system within the brain has been tied to various behaviors such as mood and anxiety and to the biology of neuropsychiatric disorders. To investigate the link between the 5-HT system and limbic/prefrontal activity, normal subjects ($n = 26$) who underwent functional magnetic resonance imaging and faced recognition tasks were genotyped for the single nucleotide polymorphism C178T in the regulatory region of the serotonin receptor type 3 gene (*HTR3A*). We found that the subjects with C/C alleles had greater activity in the amygdala and dorsal and medial prefrontal cortices than those with C/T alleles. The C/C group also showed a faster reaction time during the task than the C/T group. The temperamental predisposition of the subjects had a significant correlation with brain activity in the C/C group. The genotype effect in the right amygdala and prefrontal cortex was largest during the first run of the experiment. These results indicate that the C178T variation in the *HTR3A* has a critical influence on the amygdaloid activity and on human face processing, probably through regulation of the receptor expression. The present study may contribute to elucidating a possible link among genes, the brain, and behavior in normal populations and may help reveal the biological basis of neuropsychiatric disorders.

Key words: fMRI; face; limbic system; reaction time; personality; habituation

Introduction

Serotonin (5-HT) plays a significant role in the biological basis of human behaviors, psychiatric diseases (Meltzer, 1990), and temperamental predisposition (Reif and Lesch, 2003). Among the several subtypes of 5-HT receptors, the type 3 receptor (5-HT_3) uniquely belongs to the superfamily of ligand-gated ionotropic receptors (Turner et al., 2004) and relates with cognitive and emotive functions in humans (Olivier et al., 2000). This may be explained by the finding that the 5-HT_3 receptor is distributed in the amygdala, hippocampus, and cingulate cortex (Tecott et al., 1993; Bloom and Morales, 1998; Morales et al., 1998). Although the precise role of this receptor subtype is still elusive, several authors have suggested that the 5-HT_3 receptor inhibits memory and learning in the amygdala (Koyama et al., 2000) and hippocampus (Staubli and Xu, 1995; Bloom and Morales, 1998) through the GABAergic inhibitory mechanism (Turner et al., 2004).

The 5-HT_3 receptor gene (*HTR3A* and *HTR3B*) has been cloned for mice (Maricq et al., 1991) and humans (Miyake et al., 1995) and assigned to a single locus at human chromosome 11 (11q23.1–23.2) (Miyake et al., 1995; Weiss et al., 1995; Bruss et al., 2000). Several authors have indicated that major psychiatric disorders such as schizophrenia (Craddock and Lendon, 1999) and affective disorder (Detera-Wadleigh et al., 1998; Baysal et al., 2002) are associated with a chromosome abnormality involving distal 11q. In an *in vitro* study, a single nucleotide polymorphism (SNP), C178T, in the upstream regulatory region of the *HTR3A* gene has been found to regulate receptor expression by affecting the translation rate of HTR3A (Niesler et al., 2001). The authors suggested that the less common T allele was related to an increase of HTR3A expression compared with the more common C allele (Niesler et al., 2001). Recently, two studies demonstrated that the C178T polymorphism is relevant to the biological mechanisms of affective disorder (Nielser et al., 2001) and the personality trait of harm avoidance (HA) (Melke et al., 2003). Therefore, it is particularly important to investigate the functional relevance of this SNP and brain activity in normal human subjects.

For this purpose, 26 subjects who underwent functional mag-

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Table 1. Demographic characteristics, personality/depression score, and 5-HTTLPR genotype of the C/C and C/T groups

C178T genotype	C/C	C/T
Number of subjects	15	11
Sex (male/female)	6/9	7/4
Mean age	23.4 (3.8)	21.6 (2.1)
Education (years)	15.8 (1.5)	15.6 (1.2)
TCI		
HA	12.0 (4.8)	11.1 (4.3)
NS	9.8 (4.1)	9.9 (4.1)
RD	9.5 (1.9)	9.9 (2.8)
Persistence	2.4 (1.1)	2.6 (1.2)
BDI	3.9 (4.1)	4.0 (4.6)
5-HTTLPR	s/s, 10; s/l, 5	s/s, 4; s/l, 5; l/l, 2

Values are the mean and SD of each group. The ethnicity of the subjects is Japanese. 5-HTTLPR, Number of subjects in each genotype.

netic resonance imaging (fMRI) and a face recognition task were genotyped, and the blood oxygen level-dependent (BOLD) signal changes were compared between groups with different genotypes. During the task, pictures of unfamiliar faces with neutral expressions or pictures of houses were presented. The subjects were instructed to determine whether the picture was of a face or house. Face recognition was used because this task has been shown to predominantly activate the amygdala in normal subjects (for review, see Zald, 2003). The subject's personality trait as measured by the Temperament and Character Inventory (TCI) scale (Cloninger, 1987) was examined in relation to the brain activity and genotype. We predicted that the signals in the amygdala would be reduced in subjects with a T allele, because this allele is associated with increased receptor expression (Nielser et al., 2001) and subsequently involves the GABAergic inhibitory mechanism (Turner et al., 2004). Furthermore, responses in the human amygdala have been shown to change across experimental runs (Breiter et al., 1996). In the present study, the experimental run was repeated three times to investigate the genetic modulation of habituation in amygdaloid activation.

Materials and Methods

Subjects. Twenty-six right-handed healthy subjects participated in the experiment after providing written informed consent. Demographic characteristics of the subjects are summarized in Table 1. The study was approved by the ethics committee at the National Institute for Physiological Sciences and Fujita Health University and by the Institutional Review Board at the Nagoya University Graduate School of Environmental Studies. The subjects completed the Japanese versions of the Beck Depression Inventory (BDI) (Beck and Steer, 1993) and the TCI (125 items) (Cloninger, 1987)

Genotyping of C178T: PCR-restriction fragment length polymorphism. A blood sample was collected from each subject, and genomic DNA was extracted using standard methods. The primer pairs were 5′-TTTCCTCCCGCCTGAAAC-3′ and 5′-AAGTCCTGCTGCTTCCCG-3′. DNA amplification was performed using an iCycler (Bio-Rad Laboratories Japan, Tokyo, Japan). The reaction mixture was in a 6 μl volume containing 10 ng of sample DNA, a 0.25 mM concentration of each primer, and 200 mM each of deoxy NTP, 1× PCR Gold buffer, 2 mM $MgCl_2$, 5% DMSO, and 0.15 U of AmpliTaq Gold (Applied Biosystems Japan, Tokyo, Japan). Initial denaturation at 95°C for 9 min was followed by 45 cycles of denaturation at 95°C for 15 s, primer annealing at 57°C for 20 s, and primer extension at 72°C for 30 s, with a final extension reaction at 72°C for 7 min. Then, 6 μl of the PCR product was digested with 1 U of restriction enzyme, Hpy188III, according to the manufacturer's recommendations. DNA fragments were resolved by electrophoresis in a 6% acrylamide gel stained with ethidium bromide. In addition to the C178T polymorphism of HTR3A, the subjects were genotyped for the 5-HTTLPR polymorphism according to the method described by Lesch et al. (1996).

Experimental procedure. Digitized grayscale pictures of 48 faces with neutral expressions taken from posers (24 males and 24 females) and pictures of 20 houses created using computer graphics software (Aska-Pro; Logic, Kanazawa, Japan) served as the stimuli (Iidaka et al., 2001). These pictures were divided into two sets of stimuli, which were assigned randomly to each subject. For each face picture, an inverted version and inverted-blurred version were created with equal luminance using commercial photographic software. In each run, 24 upright faces, 24 inverted faces, 24 inverted-blurred faces, 10 houses, and 24 null events with fixation were presented randomly. Each run was repeated three times with the same set of stimuli intermixed randomly. Although in the original experiment the inverted and inverted-blurred face conditions were included, the results for these conditions are not reported here. Hereafter, we will refer to the "upright face" condition as the "face" condition. Each subject saw these stimuli successively, one at a time, for 500 ms with an interstimulus interval of 4500 ms. During the interval, a fixation point was shown. In the fMRI experiment, the stimuli were projected onto a transparent screen hanging on the bore of a magnet 75 cm from the subject's eyes. The subjects viewed the stimuli through a tilted mirror attached to the head coil of the scanner. All of the stimuli subtended a visual angle of ~5.5 × 7°. The subject was asked to judge whether the presented stimulus was a face or a house and press a corresponding button with his or her right hand. The reaction time (RT) for the task during the fMRI experiment was compared between genotypes using one-way ANOVA and is plotted in Figure 1.

fMRI data acquisition and analysis. Functional images of the entire brain were acquired in an axial orientation using a 3 tesla Siemens (Erlangen, Germany) Allegra MRI scanner equipped with single-shot echo planar image [EPI; repetition time (TR), 2.3 s; echo time (TE), 30 ms; flip angle, 80°; 64 × 64 matrix; 36 slices; voxel size, 3 × 3 × 3 mm] sensitive to BOLD contrast (Ogawa et al., 1992). After discarding the first 6 images, the remaining 234 successive images in each run were subjected to analysis. A high-resolution anatomical T1-weighted image was also acquired (MPRAGE; TR, 2.5 s; TE, 4.38 ms; flip angle, 8°; 256 × 256 matrix; 192 slices; voxel size, 0.75 × 0.75 × 1 mm) for each subject. The fMRI experiment was controlled using Presentation software (Neurobehavioral Systems, Albany, CA).

Data were analyzed by SPM99 (Wellcome Department of Imaging Neuroscience, London, UK). First, all volumes were realigned spatially to the final volume, and the signal in each slice was realigned temporally to that obtained in the middle slice using a sinc interpolation. The resliced volumes were normalized to the MNI (Montreal Neurological Institute) space (Evans et al., 1993) using a transformation matrix obtained from the normalization process of the mean EPI of each individual subject to the EPI template. The normalized images were spatially smoothed with an 8 mm Gaussian kernel.

After preprocessing, statistical analysis of each individual subject was conducted using the general linear model (Friston et al., 1995). At the first level, each single event was modeled as a hemodynamic response function and its temporal derivative. Low-pass and high-pass frequency filters were applied to the time-series data. For each subject, motion parameters were included as regressors of no interest to take into account the effects of head motion not removed at the realignment stage. The images were scaled to a grand mean of 100 over all voxels and scans within each session. In the subtraction analysis, five conditions (correct responses for the four experimental conditions and incorrect responses) were modeled separately. Parameter estimates for each condition and for the difference between the conditions were calculated from the least-mean-square fit of the model to the time-series data. Our particular interest was in the images of parameter estimates representing the difference in event-related activity between the face condition and house condition.

At the second-level (random-effects) analysis, we compared the brain activity between genotypes by entering contrast images of each individual subject containing parameter estimates representing activation for the face minus house condition into a two-sample t test. The statistical threshold of the analysis was set at $p = 0.001$, uncorrected for multiple

comparisons for height; clusters were >5 voxels. This height threshold was chosen because the difference in amygdaloid activity between the genotypes was predicted as noted in the Introduction. Region names, coordinates, and *Z* values for the difference between the genotypes are tabulated in Table 2. Amygdaloid and prefrontal regions with a significant group difference are shown in Figures 2 and 3, respectively. Signals extracted from the four regions with a significant group difference are plotted in Figure 4. These signals were extracted from the spherical regions of interest that were drawn on each region with a radius of 8 mm using MarsBaR software (Brett et al., 2002). As supplementary analyses, the subject's age or sex (male or female as a categorical variable) was included in the analysis of covariance to exclude the effect of these factors from the results for group differences.

Table 2. Brain regions with significant difference in activation between the genotype

	Region name	R/L	Voxel size	*Z* value	Coordinate
C/C > C/T					
	Middle frontal gyrus (BA9)	R	17	3.82	44, 30, 32
	Intraparietal sulcus (BA7)	L	11	3.58	−26, −40, 52
	SMA (BA6)	R	14	3.50	4, −22, 48
	Amygdala	R	9	3.48	22, −4, −30
	Superior frontal gyrus (BA10)	R	16	3.45	20, 58, 24
C/T > C/T					
	Fusiform gyrus (BA37)	R	27	3.42	38, −62, −30

R, Right hemisphere; L, left hemisphere; SMA, supplementary motor area.

To examine the relationship between the temperamental predisposition, genotype, and brain responses, multiple regression analyses were conducted using the score of the TCI subscale, the signal change in the amygdala and prefrontal areas, and the genotype as variables (Hariri et al., 2005). Significance of the interaction effect of genotype and BOLD signal on the TCI subscale was tested at the $p = 0.05$ level. The correlations between the right amygdala activity and HA score, between the right superior frontal gyrus activity and novelty-seeking (NS) score, and between the medial prefrontal activity and reward dependence (RD) score are plotted in Figure 6.

To test whether the temporal pattern of activation differs between the genotypes, the contrast image of the face minus house condition was created separately for each run. Separate ANOVAs with the RT as a covariate were conducted for the first, second, and third runs at the threshold of $p = 0.001$, uncorrected, and k = 10 voxels. The analysis was restricted to the right amygdala and prefrontal cortices where significant group difference was observed. Signal changes during each run were extracted from the right amygdala and superior frontal gyrus and are shown in Figure 7. The results shown in Tables 1 and 2 and in Figures 1–6 are from the analyses collapsing the three experimental runs.

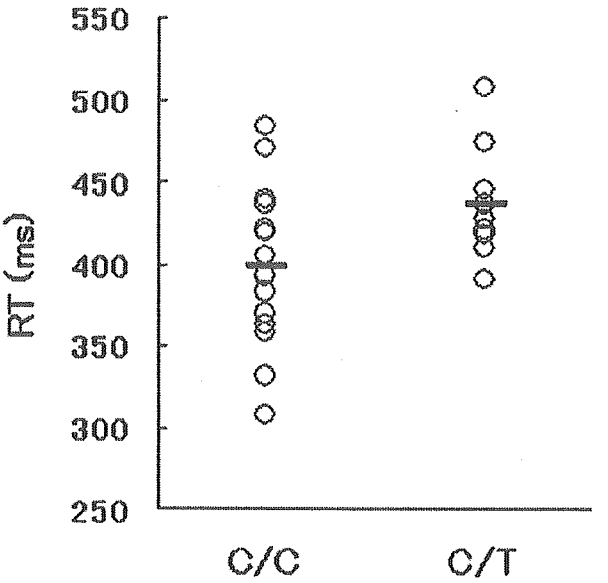

Figure 1. Reaction time (RT) during the fMRI experiment in each individual subject and group; the mean value (horizontal bar) is shown. The C/C group had a significantly faster mean RT than the C/T group.

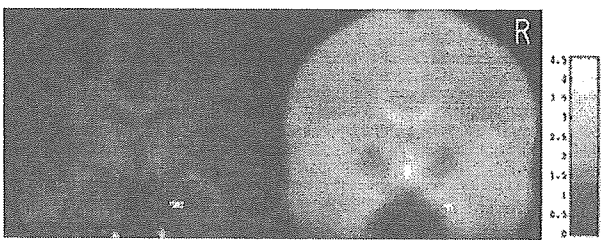

Figure 2. The right amygdala with significant group differences in activation measured by fMRI is superimposed on a high-resolution T1-weighted image of a single subject (left) and on a mean EPI of 26 subjects (right). Coronal images are shown at $y = -4$ mm. In this region, the signal was greater for the C/C group than for the C/T group. For coordinates, voxel size, and *Z* values, see Table 2. R, Right.

Results

Genomic data

For the C178T polymorphism, 15 subjects had C/C alleles and 11 subjects had C/T alleles (Table 1). The genotypes were distributed according to the Hardy-Weinberg equilibrium. These two groups did not differ significantly in terms of mean age (Kruskal–Wallis test; $p = 0.24$), sex ($\chi^2 = 1.41$; $p = 0.23$), or years of education ($F = 0.02$; $p = 0.88$). The proportion of each of the 5-HTTLPR variants did not differ significantly between the C/C and C/T groups ($\chi^2 = 4.05$; $p = 0.13$).

Behavioral data

The mean RT and accuracy for the face recognition task during the fMRI experiment were 413 ± 46 ms and 97 ± 5%, respectively. One-way ANOVA showed that the main effect of the genotype on the mean RT was significant ($F = 5.26$; $p = 0.03$). The subjects with C/C alleles had faster RTs than those with C/T alleles (398 ± 48 vs 437 ± 31 ms), as plotted in Figure 1. There was no significant group difference in accuracy during the fMRI experiment ($F = 1.83$; $p = 0.18$). Neither the mean of the BDI score nor that of the four TCI subscales differed significantly between the genotypes (Table 1).

Neuroimaging data

There were several brain regions where the signal change in response to face stimuli compared with house stimuli was significantly greater for the subjects with C/C alleles than for those with C/T alleles. These regions were located in the right amygdala (Fig. 2), right prefrontal cortex (Fig. 3), supplementary motor area, and left intraparietal sulcus (Table 2). A cluster in the right fusiform gyrus showed greater activation in the C/T group than in the C/C group. These six regions survived small volume correction for height within an 8 mm sphere at the $p = 0.05$ level. The regions also remained significant ($p = 0.001$, uncorrected) when the subject's age or sex was included in the analysis as a nuisance covariate. The degree of activation in the peak voxel listed in

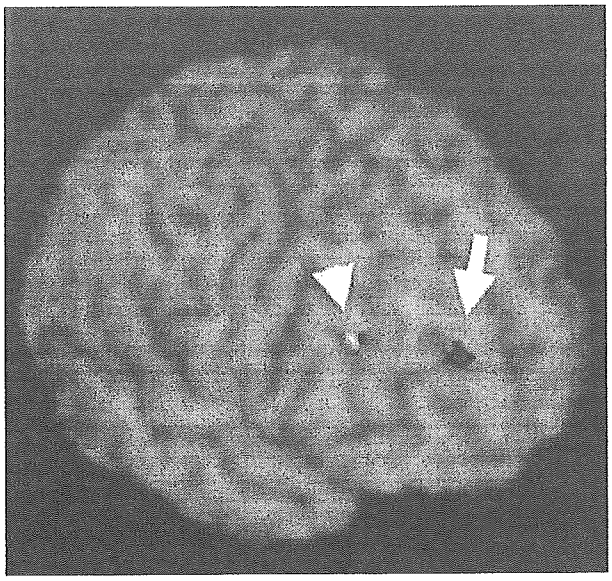

Figure 3. The areas on the right superior frontal gyrus (BA10; arrow) and middle frontal gyrus (BA9; arrowhead) where the signal was greater for the C/C group than for the C/T group are rendered on the surface of a single subject's brain. For coordinates, voxel size, and *Z* values, see Table 2.

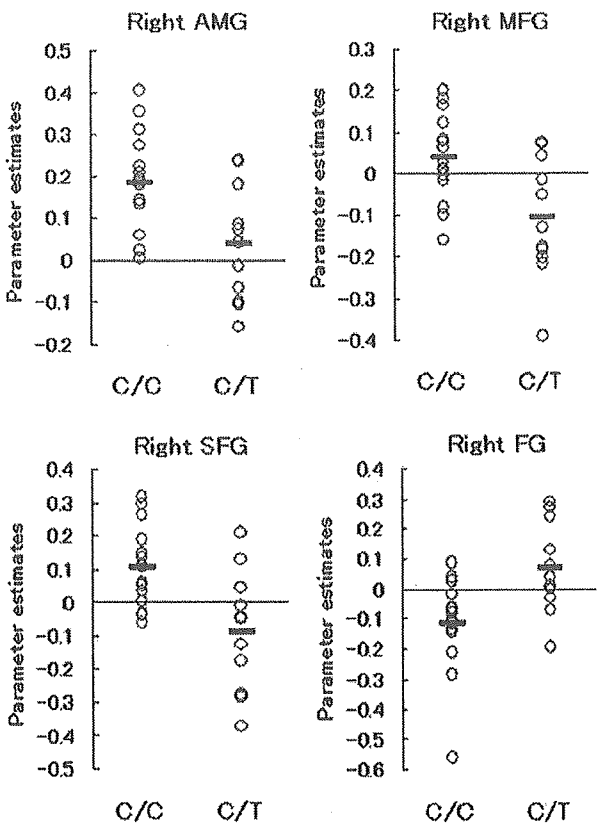

Figure 4. Signal changes in response to face stimuli compared with house stimuli in each subject extracted from spherical ROIs ($r = 8$ mm) at the right amygdala (AMG), middle frontal gyrus (MFG; BA9), superior frontal gyrus (SFG; BA10), and fusiform gyrus (FG; BA37) are shown. The horizontal bar indicates the group mean.

Table 2 did not differ between male and female subjects in either the C/C or C/T group at a lenient threshold ($p = 0.05$, uncorrected). The signals extracted from four regions in each individual subject are plotted in Figure 4. When two subjects with the I/I genotype of the 5-HTTLPR variant were excluded from the analysis, the six regions listed in Table 2 showed significant difference in activation between the C178T genotypes ($p = 0.001$, uncorrected). Therefore, the effects of C178T variants on activity in the amygdala and other regions do not simply reflect the influence of the 5-HTTLPR genotype.

In addition to these regions, we were particularly interested in the medial prefrontal cortex [mPFC; Brodmann's area 9 (BA9)] (Fig. 5, $x, y, z = -10, 52, 32$), which survived the height threshold ($z = 3.14$; $p = 0.001$, uncorrected) but not the extent threshold (k = 3 voxels), because this region has reciprocal connection with the amygdala (Ongur and Price, 2000). As shown in the right side of Figure 5, the mPFC activity positively correlated with the amygdaloid activity across all 26 subjects, suggesting that these two regions may have a functional relationship during the face recognition task. To examine whether the functional coupling of the amygdala and mPFC would be modulated by genotype or experimental run, the correlation coefficient was computed separately for each run and genotype. A positive correlation was significant only during the first ($r = 0.58$; $p < 0.05$) and second ($r = 0.58$; $p < 0.05$) runs in the C/C group.

The functional relationship between the temperamental predisposition as measured by the TCI subscale and BOLD signal is modulated by the C178T genotype as shown in Figure 6. The right amygdala activity is significantly and negatively correlated with the HA score in the C/C group ($r = -0.69$; $p = 0.004$) but not in the C/T group ($r = 0.21$; $p = 0.52$). Activity in the right superior frontal gyrus (BA10) had a significant positive correlation with the NS score in the C/C group ($r = 0.51$; $p = 0.03$) but not in the C/T group ($r = -0.41$; $p = 0.19$). Finally, the RD score significantly and negatively correlated with mPFC (BA9) activity in the C/C group ($r = -0.57$; $p = 0.02$) but not in the C/T group ($r = 0.51$; $p = 0.37$). In these cases, multiple regression analyses showed a significant genotype-by-BOLD interaction effect on the HA ($p < 0.05$), NS ($p < 0.05$), and RD ($p < 0.01$) scores.

Temporal pattern of activation

The right amygdala ($x, y, z = 26, -4, -28$) and superior frontal gyrus (BA10; $x, y, z = 18, 50, 24$) showed a significant difference in activation between the genotypes only during the first run when the difference in the RT was taken into account. As shown in Figure 7, the subjects with C/C alleles had higher signals in these regions than those with C/T alleles, particularly during the first run. We conducted two-way ANOVA with a Greenhouse-Geisser correction for the run (within subjects) and genotype (between subjects) as factors on signal and RT data. There was a significant interaction effect of the run and genotype on the BOLD signal (amygdala: $F = 3.85$, $p < 0.05$; superior frontal gyrus: $F = 4.39$, $p < 0.05$). A *post hoc t* test showed that the genotype effect was significant during the first ($p < 0.01$) and third ($p < 0.05$) runs for the amygdala and during the first run ($p < 0.01$) for the prefrontal cortex. The interaction effect of the run and genotype on the RT was not significant.

Discussion

Combined neurophysiological and genetic studies have been suggested as an important research strategy in the field of neuroscience (Begleiter et al., 1984; Porjesz et al., 2002; Hariri and Weinberger, 2003). The study by Hariri et al. (2002) showed an

association between amygdaloid activity and the 5-HT transporter gene had an impact on research into mood disorders, because 5-HT is known to be related to depression and stressful life events (Caspi et al., 2003). The present study, in a similar vein as their report, investigated whether the SNP, C178T, in the upstream regulatory region of the *HTR3A* gene would modulate brain activity in the limbic areas during a face recognition task. There were significant differences in the BOLD signals measured in the right amygdala and prefrontal cortices between the subjects with C/C alleles and those with C/T alleles. These effects were not simply a reflection of the 5-HTTLPR variants of the subjects. The two groups of subjects also differed in the RT obtained during the fMRI experiment. In addition, the genetic effect on the relationship between the temperament and BOLD signal was significant. In the right amygdala and prefrontal regions, the temporal pattern of activation across runs was modulated by genotype. The present findings indicate a possible role of this genetic polymorphism in face processing through neural responses in the amygdala and prefrontal system, which underlie complex human behaviors.

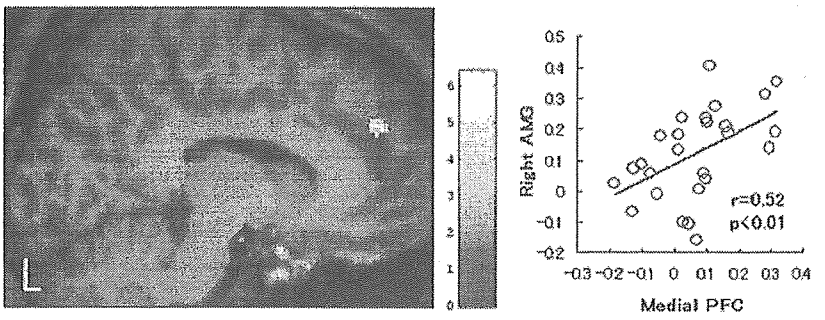

Figure 5. Left, mPFC (BA9; $x, y, z = -10, 52, 32$) where the signal was significantly greater for the C/C group than for the C/T group. A cluster is superimposed on a high-resolution T1-weighted image ($x = -10$ mm). The height threshold is set at $p = 0.005$, uncorrected for multiple comparisons for the purpose of presentation. Right, Significant positive correlation between right amygdaloid activity and medial prefrontal activity. A regression line, a correlation coefficient, and a p value are also shown. AMG, Amygdala; PFC, prefrontal cortex.

The group fMRI analysis clearly showed that the degree of activation in the right amygdala differed between the subjects with C/C alleles and those with C/T alleles. Changes in the BOLD signal imply changes in the local concentration of oxy-/deoxy-hemoglobin (Ogawa et al., 1992), but not the direct synaptic activity associated with the release and the receptor binding of particular neurotransmitters. However, several findings indicate that the genetic polymorphism of the 5-HT_3 system may alter hemodynamic responses in specific regions of the human brain during cognitive tasks. First, the 5-HT_3 receptor is preferentially expressed in the amygdala and the cingulate gyrus in animals and humans (Tecott et al., 1993; Bloom and Morales, 1998; Morales et al., 1998). Second, in an *in vitro* study, the T allele of the C178T polymorphism has been suggested to regulate receptor expression by enhancing the translation rate of the downstream HTR3A (Niesler et al., 2001). Third, a positron emission tomographic study showed that administration of the 5-HT_3 receptor antagonist altered the amygdaloid blood flow in human subjects (Berman et al., 2002). Finally, *in vitro* studies found that the specific 5-HT_3 receptor agonist facilitates inhibitory GABA release in the amygdala (Koyama et al., 2000), hippocampus (Turner et al., 2004), and other areas (Bloom and Morales, 1998). These results may suggest that the right amygdaloid function in the subjects with a T allele is inhibited by enhanced receptor expression and GABAergic neurotransmission.

In addition to the limbic regions, two dorsolateral prefrontal cortices had greater activation in the C/C group than in the C/T group. These areas are particularly involved in the processes of working memory and episodic encoding of nonverbal materials such as human faces (Kelley et al., 1998). Higher-order operations on facial information in the prefrontal cortex may be conducted extensively in the subjects with C/C alleles. In contrast, activity in the right fusiform gyrus, a region that is sensitive to the human face (Allison et al., 1994), was greater in the C/T group than in the C/C group. This may be as a result of the subjects with the T allele relying more heavily on the visual cortical pathway to process the stimuli than the other subjects, who used the amygdala-mediated subcortical route. Strong right-lateralization of activation would be attributable to that the right hemisphere is more specialized for face processing in human subjects than the left hemisphere (Sergent and Bindra, 1981).

A novel finding of the present study is that the relationship between the TCI score and brain response was modulated by genotype, although the mean TCI score did not differ between the two groups. A significant negative correlation between HA and amygdaloid activity may imply that enhanced 5-HT function in those with a high HA score reduced neural response in the limbic region in the C/C group (Del-Ben et al., 2005). A positive correlation between activity in the superior frontal gyrus and the NS in the C/C group may relate to that the subjects with high extraversion scores having greater prefrontal activation during working memory tasks than those with low extraversion scores (Kumari et al., 2004). Finally, the correlation between the medial prefrontal activity and RD may be underpinned by the evidence that the medial prefrontal–limbic circuit subserves reward processing in experimental animals (Wise, 2000).

The RT during the fMRI experiment differed between the genotypes, indicating that the subjects with the T allele do not process face stimuli as fast as those who are homozygous for the C allele. Amygdaloid function has been implicated in conditioned associative learning (Ono et al., 1995) and in the processing of stimuli with biological significance in multiple sensory domains (Zald, 2003). The amygdala is a part of the subcortical information pathway that bypasses the primary visual cortex to respond rapidly to salient stimuli (Morris et al., 1999; Adolphs, 2002). The present results may suggest, in accordance with this model, that a shorter mean RT in the C/C group is a reflection of activation in the amygdala-mediated subcortical pathway. The result that the C/T group, which used the right fusiform gyrus during the task, responded more slowly than the C/C group fits with this hypothesis.

Neuroimaging studies in normal subjects have shown that the amygdaloid and prefrontal responses to face stimuli are largest during the initial phase of the experiment (Breiter et al., 1996; Wright et al., 2001). In contrast, an fMRI study showed that the peak of the amygdaloid response in depressed subjects occurred later in the time course than that in control subjects (Siegle et al., 2002). Therefore, we hypothesized that the genotype effect in the amygdala would be found predominantly during the first run of the experiment. As shown in Figure 7, the difference in activation between the groups was largest during the first run in the right amygdala and superior frontal gyrus. The C/C group showed a

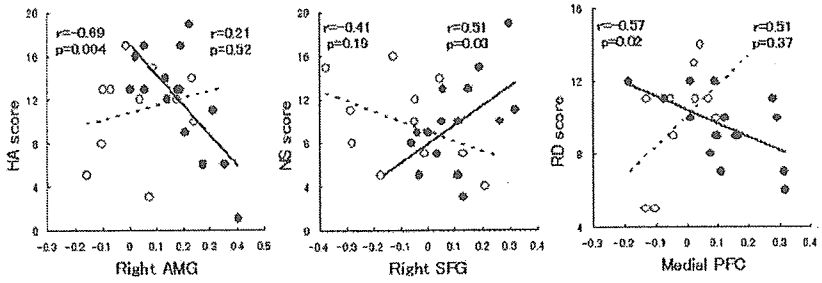

Figure 6. Correlations between the TCI subscale score and the brain activity are plotted separately for each genotype group. Left, Harm avoidance (HA) score and right amygdaloid activity. AMG, Amygdala. Middle, Novelty-seeking (NS) score and activity in the right superior frontal gyrus (SFG; BA10). Right, Reward dependence (RD) score and medial prefrontal cortex (PFC; BA9) activity. Filled circles indicate subjects with C/C alleles, and open circles indicate subjects with C/T alleles. The regression lines (C/C group, solid line; C/T group, dotted line), correlation coefficients, and p values are also shown.

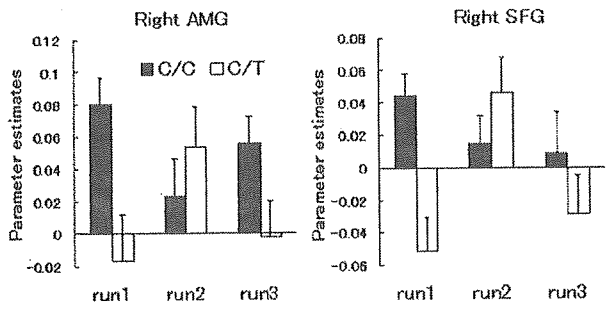

Figure 7. The mean and SE of the right amygdala (AMG; $x, y, z = 24, -4, -30$) and prefrontal [superior frontal gyrus (SFG); BA10; $x, y, z = 18, 52, 24$] responses to face stimuli in each group of subjects during the first (run1), second (run2), and third (run3) runs of the fMRI experiment are shown. ■, C/C group; □, C/T group.

typical temporal pattern of activation, whereas in the C/T group, the peak was observed in the second run. Reduced activation and the differential habituation pattern would be related to disturbance in stress hormone response through the 5-HT system (Van Praag, 2004).

Another significant finding was that the mPFC activation was greater in the C/C group than in the C/T group and positively correlated with the amygdaloid activation. Functional coupling of mPFC and the amygdala was significant during the first and the second runs in the subjects with C/C alleles. The mPFC plays a role in reward processing and goal-directed behavior in animals (Wise, 2000) and in humans (Rogers et al., 2004) and exerts significant control over the emotional–cognitive aspect of behavior (Vertes, 2004). Neuroanatomical studies in animals have shown that the mPFC has reciprocal connections with several nuclei of the amygdala (Ongur and Price, 2000), thalamus, and brainstem (Vertes, 2004) and relates with hormonal response to stress through the hypothalamic–pituitary–adrenal axis (Crane et al., 2003).

The present results have clinical implications in neuropsychiatry, because the C178T variant was more frequently found in bipolar affective patients than in a control group (Nielser et al., 2001). Differential BOLD signals and blood flow activation in the region of the amygdala, compared with normal subjects, have been observed in patients with mood disorders (Drevets, 2003) and anxiety disorders (Anand and Shekhar, 2003). An increased stress hormone level found in patients with depression and an anxiety disorder (Van Praag, 2004) is a biological marker of susceptibility to socio-psychological stress. Therefore, it is suggested that the subjects with the T allele and an increased 5-HT_3 receptor expression (Nielser et al., 2001) may have an elevated hormonal level because the 5-HT_3 receptor is related to ACTH secretion in response to acute stress (Bhatnagar et al., 2004).

There are several caveats in the present study. First, the number of subjects is small and differed between the groups. In the future, a study with a larger number of subjects should be conducted. Second, the amygdala activity might have been limited because we used neutral faces, which elicit less activation in this region than faces expressing emotion, as stimuli. Third, the fact that there were no significant differences in the mean TCI subscale scores or BDI score between the genotypes may have obscured the functional relevance of the amygdala to personality and emotion. However, a recent study of HTR3A polymorphism and personality traits also showed no differences in the HA scores between the C/C and C/T groups (Melke et al., 2003). Neurophysiological responses as measured by fMRI would be more sensitive than questionnaires such as the BDI and TCI for detecting group differences. Neural activation in the amygdala should be treated as an endophenotype underlying both normal and pathological brain functions (Almasy and Blangero, 2001).

In conclusion, the present study, to the best of our knowledge, revealed for the first time the significant effect of HTR3A polymorphism on the amygdala and prefrontal activity as measured by fMRI. In addition, it showed that the difference in behavioral performance during a face recognition task was related to the genetic variation, indicating a close coupling of genes, behavior, and neurophysiological responses. The present findings may have clinical implications in predicting an individual's vulnerability to socio-psychological stressors before the onset of neuropsychiatric disorders. However, the causal relationship between genotype and brain activation is not fully elucidated in the present study. Therefore, additional investigation involving *in vitro* as well as *in vivo* experiments using animals, normal volunteers, and clinical populations are needed in the future.

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Association study of the frizzled-3 (FZD3) gene with schizophrenia and mood disorders

Short Communication

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Summary. Two research groups have recently reported a significant association between schizophrenia and genetic variants of Frizzled-3 (FZD3) gene. We examined a possible association in a Japanese sample of schizophrenia, bipolar disorder, unipolar depression and controls with four single nucleotide polymorphisms (SNPs), tested in previous reports. We failed to find significant association in the four SNPs or haplotype analysis. The FZD3 gene might not play a role in conferring susceptibility to major psychosis in our sample.

Keywords: FZD3, schizophrenia, mood disorder, association study, single nucleotide polymorphism (SNP).

Introduction

Schizophrenia is a complex genetic disorder characterized by disturbances of cognition, emotion and social functioning. This disease is believed to involve genetic abnormalities in developmental/plasticity related processes during a critical period in neuronal growth (Weinberger et al., 2001). Wnt signal transduction cascades have been implicated in a variety of neurodevelopmental processes, e.g. segmentation, central nervous system patterning, and cell divisions (Wodarz and Nusse, 1998). Wnt proteins signal via cell surface transmembrane receptors, termed frizzleds, which display many properties

characteristic of members of the superfamily of G-protein-coupled receptors (Wang and Malbon, 2004). The frizzled-3 (FZD3) gene, a member of frizzles, is located on chromosome 8p21, repeatedly suggested as a positive linkage locus for schizophrenia (Lewis et al., 2003; McGuffin et al., 2003). The FDZ3 gene consists of 8 exons and 7 introns, spanning approximately 70 Kb (Kirikoshi et al., 2000). In accordance with this, two research groups have recently reported a significant association between schizophrenia and the FZD3 gene in Japanese and Chinese samples (Katsu et al., 2003; Yang et al., 2003). We tried to replicate these findings in an independent Asian sample. Furthermore, we also examined the possible association between the FZD3 gene with mood disorders, since schizophrenia and mood disorders might share the genetic vulnerability (Berrettini, 2003).

Methods and materials

Subjects

Subjects were 427 patients with schizophrenia (221 males and 206 females with mean age of 44.2 years [SD 14.5]), 91 with bipolar disorder (40 and 51; 53.6 years [SD 14.8]), and 396 with major depression (155 and 241; 53.4 years [SD 16.1]) and 473 healthy controls (228 and 245; 36.1 years [SD 12.5]). All the subjects were biologically unrelated Japanese. Consensus diagnosis was made for each patient by at least two trained psychiatrists according to the DSM-IV criteria. Controls were healthy volunteers who had no current or past contact to psychiatric services. After description of the study, written informed consent was obtained from every subject. The study protocol was approved by institutional ethical committees.

SNP genotyping

Venous blood was drawn from the subjects and genomic DNA was extracted from whole blood according to the standard procedures. We genotyped four SNPs (single nucleotide polymorphisms; dbSNP accession: rs960914 in intron3, rs2241802 in exon5: A435G, L145L, rs2323019 in intron5 and rs352203 in intron5) in the FZD3 gene, which were examined in the previous two studies (Katsu et al., 2003; Yang et al., 2003). Genotyping was performed with the TaqMan 5′-exonuclease allelic discrimination assay, described previously (Hashimoto et al., 2004a, b). Briefly, primers and probes for detection of the SNPs are: rs960914: forward primer 5′-CTTTTATAAAGAAATTTGAAACAT CAGAACATGGGA-3′, reverse primer 5′-ACTTTTTCACTGCTTGGGAGTTATTCT-3′, probe 1 5′-VIC-CTGAATGGCTGCTATC-MGB-3′, and probe 2 5′-FAM-TCTGAATGGCTACTATC-MGB-3′; rs2241802: forward primer 5′-ATGAGCCATATCCTCGACTTGTG-3′, reverse primer 5′-GGACACCAAAAACCATAGTCTCTCT-3′, probe 1 5′-VIC-TCCAGCTAAATTCAG-MGB-3′, and probe 2 5′-FAM-CAGCCAAATTCAG-MGB-3′; rs2323019: forward primer 5′-GAAT TACTTTGTTTTTCTAGATTCTTGAATTGAAAGC-3′, reverse primer 5′-CCAACCTGGTTAA TAATGGTCTTTTGG-3′, probe 1 5′-VIC-TCATTTATTGTCAATGTTTTAA-MGB-3′, and probe 2 5′-TCATTTATTGTCAATATTTTAA-MGB-3′; rs352203: forward primer 5′-CCTGAAAAAA TATTCTATATCTCTTGTTTTGCCA-3′, reverse primer 5′-CAACCAGGACATAACAGTATTA CAGTTTCTAT-3′, probe 1 5′-VIC-TCCTTCATGTCGTATTC-MGB-3′, and probe 2 5′-FAM-TTTCCTTCATATCGTATTC-MGB-3′. PCR cycling conditions were: at 95°C for 10 minutes, 45 cycles of 92°C for 15 seconds and 60°C for 1 minute.

Statistical analysis

Statistical analysis of association studies was performed using SNPAlyse software (DYNACOM, Yokohama, Japan). The presence of Hardy-Weinberg equilibrium was examined using the $\chi\chi^2$ test for goodness of fit. Allele distributions between patients and controls were analyzed by the

Table 1. Allele distribution for SNPs in the FZD3 gene between major psychoses and controls

dbSNP ID	SNP	Controls	Schizophrenia		Bipolar		Unipolar	
		n = 473	n = 427	*P* value	n = 91	*P* value	n = 397	*P* value
rs960914	T/C	.398	.396	.91	.352	.23	.386	.61
rs2241802	G/A	.453	.458	.85	.451	.94	.455	.96
rs2323019	A/G	.407	.420	.57	.357	.21	.409	.91
rs352203	T/C	.397	.396	.97	.352	.24	.386	.64

χ^2 test for independence. The measures of linkage disequilibrium (LD), denoted as D', was calculated from the haplotype frequency using Expectation-Maximization algorithm. Case-control haplotype analysis was performed by the permutation method to obtain empirical significance (Good, 2000). The global *p*-values represent the overall significance using the χ^2 test when the observed versus expected frequencies of all the haplotypes are considered together. The individual haplotype was tested for association by grouping all others together and applying the χ^2 test with 1 df. *P*-values were calculated based on 10,000 replications. All *p*-values reported are two tailed. Statistical significance was defined at $p < 0.05$.

Results

The obtained allele frequencies for the patients and controls are shown in Table 1. The genotype distributions for all the diagnostic groups were in Hardy-Weinberg equilibrium (data not shown). There was no significant difference in genotype distributions or allele frequencies of the four SNPs in the FZD3 gene between the controls and any patient group, although previous studies reported positive associations between schizophrenia and several SNPs (Katsu et al., 2003; Yang et al., 2003). We computed the LD between the SNPs using D', which ranged between 0.8 and 1.0, indicating strong to intermediate LD between the markers. Adjacent combinations of up to four markers were tested, however, any haplotype combination was not significantly associated with any diagnostic group (all global *p*-values > 0.2).

Discussion

This study examined the possible association of the FZD gene with schizophrenia and mood disorders in our Japanese sample. We obtained no evidence for a significant association of the genetic variations of the FZD gene with any diagnostic group, suggesting that the examined polymorphisms play no major role in the pathogenesis of major psychoses in our sample. Our results are thus inconsistent with the results of the previous case-control study which reported a significantly higher frequency of the T allele of rs960914 in patients with schizophrenia than in controls (Katsu et al., 2003). The frequencies of the T allele were 0.62 and 0.51 in schizophrenics (n = 209) and controls (n = 200) in their Japanese sample, while the frequencies of the T allele were 0.60 and 0.60 in patients with schizophrenia (n = 427) and controls (n = 473) in ours. A highly significant association of the other three SNPs (rs2241802, rs2323019 and rs352203) and their three marker haplotypes with schizophrenia patients was reported in a family-based association study in a Chinese population