Neuromol Med (2009) 11:53–57
DOI 10.1007/s12017-009-8060-7

ORIGINAL PAPER

CLOCK may Predict the Response to Fluvoxamine Treatment in Japanese Major Depressive Disorder Patients

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Received: 17 February 2009 / Accepted: 17 March 2009 / Published online: 4 April 2009

Abstract Recent studies have shown that selective serotonin reuptake inhibitors (SSRIs) have circadian properties, suggesting that the antidepressive action of SSRIs may also be attributable to circadian mechanisms. Another study reported an association between clock gene (*CLOCK*) and improvements in insomnia symptoms from SSRIs treatment. Therefore, we examined the association between *CLOCK* and the efficacy of fluvoxamine treatment in 121 patients with Japanese major depressive disorder (MDD). The MDD patients in this study had scores of 12 or higher on the 17 items of the Structured Interview Guide for Hamilton Rating Scale for Depression (SIGH-D). We defined a therapeutic response as a decrease of more than a 50% in baseline SIGH-D within 8 weeks, and clinical remission as a SIGH-D score of less than seven at 8 weeks. We selected three tagging SNPs in *CLOCK* for the subsequent statistical association analysis. We detected a significant association between rs3736544, a synonymous polymorphism in exon 20, and the fluvoxamine therapeutic response in MDD in the allele/genotype-wise analyses. In addition, remission with fluvoxamine was also significantly associated with rs3736544. These associations remained significant after Bonferroni correction. Moreover, haplotype analysis findings supported these significant associations, which appeared to be due mainly to rs3736544, in the fluvoxamine therapeutic remission. Our results indicate that *CLOCK* genotype may be a predictor of fluvoxamine treatment response in Japanese MDD. However, our sample size was small, and a replication study using larger samples may be required for conclusive results.

Keywords Major depressive disorder · *CLOCK* · Tagging SNPs · Fluvoxamine · SSRIs

Introduction

Major depressive disorder (MDD) patients commonly present not only abnormalities in sleep–wake rhythms but also disruptions in biological circadian rhythms. Therefore, disruptions in circadian rhythms have been suggested to be involved in the pathogenesis of MDD (Barnard and Nolan 2008; Kishi et al. 2008a, 2008b). All psychotropic drugs act on the systems of neurotransmitters such as dopamine and serotonin in the brain (Barnard and Nolan 2008), and recently these neurotransmitter systems have been reported to have reciprocal interactions with circadian rhythms (Monteleone and Maj 2008).

Selective serotonin reuptake inhibitors (SSRIs) such as fluvoxamine, which are major therapeutic agents for MDD, inhibit serotonin transport in the presynaptic neuron, and increase the extracellular serotonin level. This mechanism is believed to relieve depressive symptoms (Peveler and

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Kendrick 2005). On the other hand, many animal and in vitro studies have shown that serotonin directly affects circadian rhythms (Monteleone and Maj 2008), and SSRIs have also been reported to have circadian properties. SSRIs have a phase shifting effect in neural firing in the rat suprachiasmatic nucleus (Sprouse et al. 2006), and change the expression of clock genes in the striatum and hippocampus of mice (Uz et al. 2005), suggesting that the antidepressive action of SSRIs may also be attributable to circadian mechanisms. Therefore, we considered that clock genes might be therapeutic targets for SSRIs.

The clock gene (*CLOCK*, OMIM *601851, 25 exons in this genomic region spanning 115.138 kb), located on 4q12, is one of the major components of the cellular clock gene mechanism. It is known to be associated with human circadian preference (morningness/eveningness) (Katzenberg et al. 1998; Mishima et al. 2005). Several clinical subgroup analyses have shown a significant association between an SNP (rs1801260: T3111C) in *CLOCK* and sleep disregulation in mood disorders including MDD and bipolar disorder (BP) (Serretti et al. 2003) and a higher recurrence rate in BP (Benedetti et al. 2003). In addition, Serretti and colleagues reported an association between T3111C and improved insomnia from fluvoxamine or paroxetine treatment (Serretti et al. 2005). However, three genetic studies, including our previous study, reported no association between *CLOCK* and MDD (Bailer et al. 2005; Desan et al. 2000; Kishi et al. 2008a). Thus, there is disagreement in the results of these studies as to treatment response and the pathophysiology of MDD (Gratacos et al. 2008).

In this study, we examined the association between *CLOCK* and the efficacy of fluvoxamine treatment in Japanese MDD patients. To do this, we applied the recently recommended strategy of "gene-based" association analysis (Neale and Sham 2004).

Materials and Methods

Subjects

The subjects were 121 MDD patients (60 males and 61 females: mean age ± standard deviation (SD) 44.5 ± 16.5 years). All subjects were unrelated to each other, ethnically Japanese, and lived in the central area of Japan. The patients were diagnosed according to DSM-IV criteria with consensus of at least two experienced psychiatrists on the basis of a review of medical records. Fluvoxamine was taken two or three times a day for 8 weeks. The initial total dose was 50–100 mg per day, and the dosage was then increased gradually to a maximum of 150 mg, depending on the patients' condition. Patients with insomnia and severe anxiety were prescribed benzodiazepine drugs, but no other psychotropic drugs were permitted during the study. The study was described to subjects and written informed consent was obtained from each. This study was approved by the Ethics Committee at Fujita Health University.

Data Collection

The MDD patients in this study had scores of 12 or higher on the 17 items of the Structured Interview Guide for Hamilton Rating Scale for Depression (SIGH-D). Patients with this moderate range of severity tend to respond to antidepressants (Saito et al. 2006). We defined a therapeutic response as a decrease of more than 50% in baseline SIGH-D within 8 weeks, and a clinical remission as a SIGH-D score of less than 7 at 8 weeks. Detailed information on data collection was described in a previous paper (Saito et al. 2006). The clinical characteristics of the patients in this study, classified according to these definitions, can be seen in Table 1.

SNPs Selection and Linkage Disequilibrium (LD) Evaluation

We selected three "tagging SNPs" (rs3736544: synonymous polymorphism in exon 20, rs1801260: 3′ untranslated region (UTR) in exon 23, rs3749474: 3′ UTR in exon 23) in *CLOCK*. Detailed information can be seen in our previous paper (Kishi et al. 2008a).

SNPs Genotyping

We used TaqMan assays (Applied Biosystems, Inc., Foster City, CA,) for all SNPs. Detailed information can be seen in our previous paper (Kishi et al. 2008a).

Statistical Analysis

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

Marker-trait association analysis was used to evaluate allele- and genotype-wise associations with the chi-square test (SAS/Genetics, release 8.2, SAS Japan Inc, Tokyo, Japan), and haplotype-wise association analysis was done with a likelihood ratio test using the COCAPHASE 2.403 program (Dudbridge 2003). Bonferroni's correction was used to control inflation of the type I error rate. Power calculation was performed using the Genetic Power Calculator (Purcell et al. 2003).

The significance level for all statistical tests was 0.05.

Table 1 Clinical characteristics of the patients in both definition groups

	N			Age (mean ± SD)	Baseline SIGH-D (avg ± SD)	Fluvoxamine dose at 8 weeks (mg/day) (avg ± SD)	Number of previous episode (avg ± SD)
	Total	Male	Female				
Overall	121	60	61	44.5 ± 16.5	20.2 ± 5.88	122 ± 40.9	1.39 ± 0.658
Clinical response group[a]							
Responders	60	31	29	44.4 ± 16.3	21.5 ± 6.19	118 ± 41.1	1.36 ± 0.574
Nonresponders	61	29	32	44.3 ± 17.3	18.8 ± 5.28	125 ± 40.7	1.43 ± 0.774
P-value	0.645			0.819	0.0145	0.391	0.480
Clinical remission group[b]							
Remitters	45	22	23	43.7 ± 15.9	19.6 ± 5.06	113 ± 43.9	1.37 ± 0.598
Nonremitters	76	38	38	45.1 ± 17.1	20.5 ± 6.34	127 ± 38.2	1.41 ± 0.715
P-value	0.722			0.750	0.750	0.101	0.856

[a] Clinical response was defined as a 50% or greater decrease in the baseline SIGH-D score

[b] Clinical remission was defined as a final SIGH-D score of less than seven

Results

The LD structures of *CLOCK* from the HapMap database were described in our previous paper (Kishi et al. 2008a). Among the clinical characteristics of the patients in this study, only one significant difference with total SIGH-D score was detected at the baseline in relation to fluvoxamine therapeutic response (*P*-value = 0.0145) (Table 1). Genotype frequencies of all SNPs were in HWE. We detected a significant association between rs3736544 and the fluvoxamine therapeutic response in MDD in the allele/genotype-wise analysis (Table 2). In addition, remission with fluvoxamine was significantly associated with rs3736544 (Table 2). Moreover, the significance of these associations remained after Bonferroni correction (Table 2). We also found an association between rs3749474 and the fluvoxamine therapeutic response in MDD in the genotype-wise analysis (*P*-value: 0.0251) (Table 2). However, this might have resulted from type I error due to multiple testing (*P*-value: 0.0752 after Bonferroni's correction) (Table 2). The haplotype-wise analysis provided evidence for a significant association that appears to be due mainly to rs3736544 in fluvoxamine therapeutic remission (Table 3).

Table 2 Association analysis of tagging SNPs in *CLOCK*

SNP[a]	Phenotype	MAF	*N*	Genotype distribution[b]			*P*-value[d]			Corrected *P*-value[d,e]	
				M/M	M/m	m/m	HWE[c]	Genotype	Allele	Genotype	Allele
rs3736544 G > T	Responders	0.267	60	30	28	2	0.135				
	Nonresponders	0.115	61	48	12	1	0.804	**0.00434**	**0.00261**	**0.00130**	**0.00738**
	Remission	0.289	45	21	22	2	0.203				
	Nonremission	0.132	76	57	18	1	0.751	**0.00651**	**0.00257**	**0.0195**	**0.00771**
rs1801260 T > C	Responders	0.133	60	46	12	2	0.297				
	Nonresponders	0.189	61	39	21	1	0.328	0.187	0.243		
	Remission	0.156	45	33	10	2	0.301				
	Nonremission	0.164	76	52	23	1	0.378	0.390	0.855		
rs3749474 T > C	Responders	0.417	60	19	32	9	0.452				
	Nonresponders	0.336	61	27	27	7	0.949	0.358	0.196		
	Remission	0.467	45	12	24	9	0.632				
	Nonremission	0.322	76	34	35	7	0.637	0.0734	**0.0251**		0.0752

[a] major allele > minor allele

[b] M: major allele, m: minor allele

[c] HWE: Hardy–Weinberg equilibrium

[d] Bold numbers represent significant *P*-value

[e] Caluculated by Bonferroni's correction

Table 3 Haplotype-wise analysis of tagging SNPs in *CLOCK*

Common haplotypes rs3736544-rs1801260-rs3749474	Phenotype	Individual haplotype frequency	Individual *P*-value[a]	Phenotype	Global *P*-value[a]
GTT	Responders	0.600	0.173		
	Nonresponders	0.686		Responders	0.436
	Remission	0.548	**0.0191**	Nonresponders	
	Nonremission	0.703		Remission	**0.015**
GCC	Responders	0.146	0.401	Nonremission	
	Nonresponders	0.188			
	Remission	0.167	1.00		
	Nonremission	0.167			
TTC	Responders	0.255	**0.0137**		
	Nonresponders	0.125			
	Remission	0.286	**0.00417**		
	Nonremission	0.130			

[a] Bold numbers represent significant *P*-value

Discussion

In this study, we detected a significant association between rs3736544 in *CLOCK*, which is a synonymous polymorphism in exon 20, and the fluvoxamine therapeutic response and remission in the allele/genotype-wise analysis. This significance remained after Bonferroni correction. Haplotype analysis indicated three common haplotypes (rs3736544-rs1801260-rs3749474: GTT, GCC and TTC). Among them, the TTC haplotype was less prevalent in subjects with a fluvoxamine therapeutic response ($P = 0.0137$) and was associated with remission on fluvoxamine ($P = 0.00417$). The GTT haplotype was also significantly associated with remission on fluvoxamine ($P = 0.0191$). In a recent study, we selected six tagging SNPs among 106 SNPs covering all of *CLOCK*, including 5′-flanking regions about 2 kb upstream (5′) from the initial exon and about 5 kb downstream (3′) from the last exon (HapMap database contig number chr4: 55990340.. 56108588), with the criteria of an r^2 threshold greater than 0.8 in "pair-wise tagging only" mode using the Tagger program. LD structures of *CLOCK* from the HapMap database were described in our previous paper (Kishi et al. 2008a). However, the LD structure of *CLOCK* in our sample was very tight except for rs1801260 and rs3749474 (Kishi et al. 2008a). Also, the LD structures of MDD samples treated with fluvoxamine and control samples were almost the same (Kishi et al. 2008a). As these results show, rs3736544 covers a wide and important region including the exons and the promoter region in *CLOCK*. Therefore, it is possible that rs3736544 influences biological function in the brain. In previous genetic analyses of *CLOCK*, only T3111C (rs1801260) was selected. T3111C (rs1801260) has been detected at position 3111 in the *CLOCK* mRNA 3′ untranslated region, and was reported to be associated with a substantial delay in preferred timing for activity and sleep in a human study (Katzenberg et al. 1998). As for function, T3111C (rs1801260) has been speculated to affect mRNA (Katzenberg et al. 1998); however, one study with luciferase reported no significant effect on mRNA translatability from T3111C (Robilliard et al. 2002). We found an association of rs3736544 but not T3111C (rs1801260) with treatment outcome in this study. These findings suggest that functional analyses for other regions of the *CLOCK* should be performed in future studies.

A subgroup analysis has shown a significant association between an SNP (rs1801260: T3111C) in *CLOCK* and sleep disregulation in mood disorders (Serretti et al. 2003). Because benzodiazepine drugs are surely effective for insomnia and severe anxiety in MDD patients, which might mask the sleep disruption in MDD due to circadian abnormality, the analysis which takes the usage of benzodiazepines into account may also need to be carried out in the future. Because we had only a few MDD fluvoxamine treatment samples without benzodiazepine drugs, and we wanted to avoid statistical error, we did not perform such an analysis among these samples. Another subgroup analysis has shown a higher recurrence rate in BP in relation to T3111C (Benedetti et al. 2003), but we lacked data on recurrence in our sample, so we could not perform such analysis.

Our recent study found no association between *CLOCK* and MDD in the Japanese population (Kishi et al. 2008a). Thus, there is disagreement in the results among studies as to the treatment response and the pathophysiology of MDD (Gratacos et al. 2008).

A few points of caution should be noted in interpreting our results. First, it will be necessary to investigate the possibility that rs3736544 reflects biological function,

which we did not do in the present study. Second, we did not include a mutation scan to detect rare variants with functional effects. However, it is difficult to evaluate the association of such extremely rare variants (e.g., minor allele frequencies less than 0.01) from the viewpoint of power. Third, our sample sizes were small. A replication study using larger samples may be required for conclusive results.

In conclusion, our results indicate that *CLOCK* may be associated with fluvoxamine treatment outcome in Japanese MDD.

Acknowledgments We thank Ms. M. Miyata and Ms. S. Ishihara for their technical support. This work was supported in part by research grants from the Japan Ministry of Education, Culture, Sports, Science and Technology, the Ministry of Health, Labor and Welfare, and the Health Sciences Foundation (Research on Health Sciences focusing on Drug Innovation).

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Schizophrenia Research 110 (2009) 140–148

Contents lists available at ScienceDirect

Schizophrenia Research

journal homepage: www.elsevier.com/locate/schres

Meta-analysis of association between genetic variants in *COMT* and schizophrenia: An update

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ARTICLE INFO

Article history:
Received 28 October 2008
Received in revised form 14 February 2009
Accepted 22 February 2009
Available online 28 March 2009

Keywords:
Schizophrenia
COMT
Linkage disequilibrium
Meta-analysis

ABSTRACT

A common functional polymorphism, Val108/158Met (rs4680), and haplotypes rs737865–rs4680–rs165599 in the Catechol-O-methyltransferase gene (*COMT*) have been extensively examined for association to schizophrenia; however, results of replication studies have been inconsistent. The aim of this study was to comprehensively evaluate the genetic risk of *COMT* for schizophrenia.

First, we performed a mutation scan to detect the existence of potent functional variants in the 5′-flanking and exon regions. Second, we conducted a gene-based case–control study between tagging single nucleotide polymorphisms (SNPs) in *COMT* [19 SNPs including six possible functional SNPs (rs2075507, rs737865, rs4680, rs165599, rs165849)] and schizophrenia in large Japanese samples (schizophrenics 1118, controls 1100). Lastly, we carried out a meta-analysis of 5 functional SNPs and haplotypes (rs737865–rs4680–rs165599).

No novel functional variant was detected in the mutation scan. There is no association between these tagging SNPs in *COMT* and Japanese schizophrenia. In this updated meta-analysis, no evidence was found for an association between Val108/158Met polymorphisms, rs6267, rs165599, and haplotypes (rs7378655–rs4680–rs165599) and schizophrenia, although rs2075507 and rs737865 showed trends for significance in allele-wise analyses ($P=0.039$ in a multiplicative model, $P=0.025$ in a recessive model for rs2075507, $P=0.018$ in a dominant model for rs737865, uncorrected). This significance did not remain, however, after correcting the *P*-values using a false discovery rate controlling procedure.

Our results suggest that the *COMT* is unlikely to contribute to susceptibility to schizophrenia.

1. Introduction

The gene encoding Catechol-O-methyltransferase (*COMT*) is considered to be a likely candidate gene for schizophrenia owing to 1) the role of the enzyme in dopamine metabolism and to 2) its chromosomal location, 22q11, which has been implicated in schizophrenia by several linkage studies (Owen et al., 2005), as well as by its deletion in Velo-cardio-facial syndrome, in which patients frequently develop psychotic disorders including schizophrenia (Murphy et al., 1999).

Most *COMT* genetic association studies have focused on a particular single nucleotide polymorphism (SNP) that results in a change from Valine to Methionine at codon 158/108

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doi:10.1016/j.schres.2009.02.019

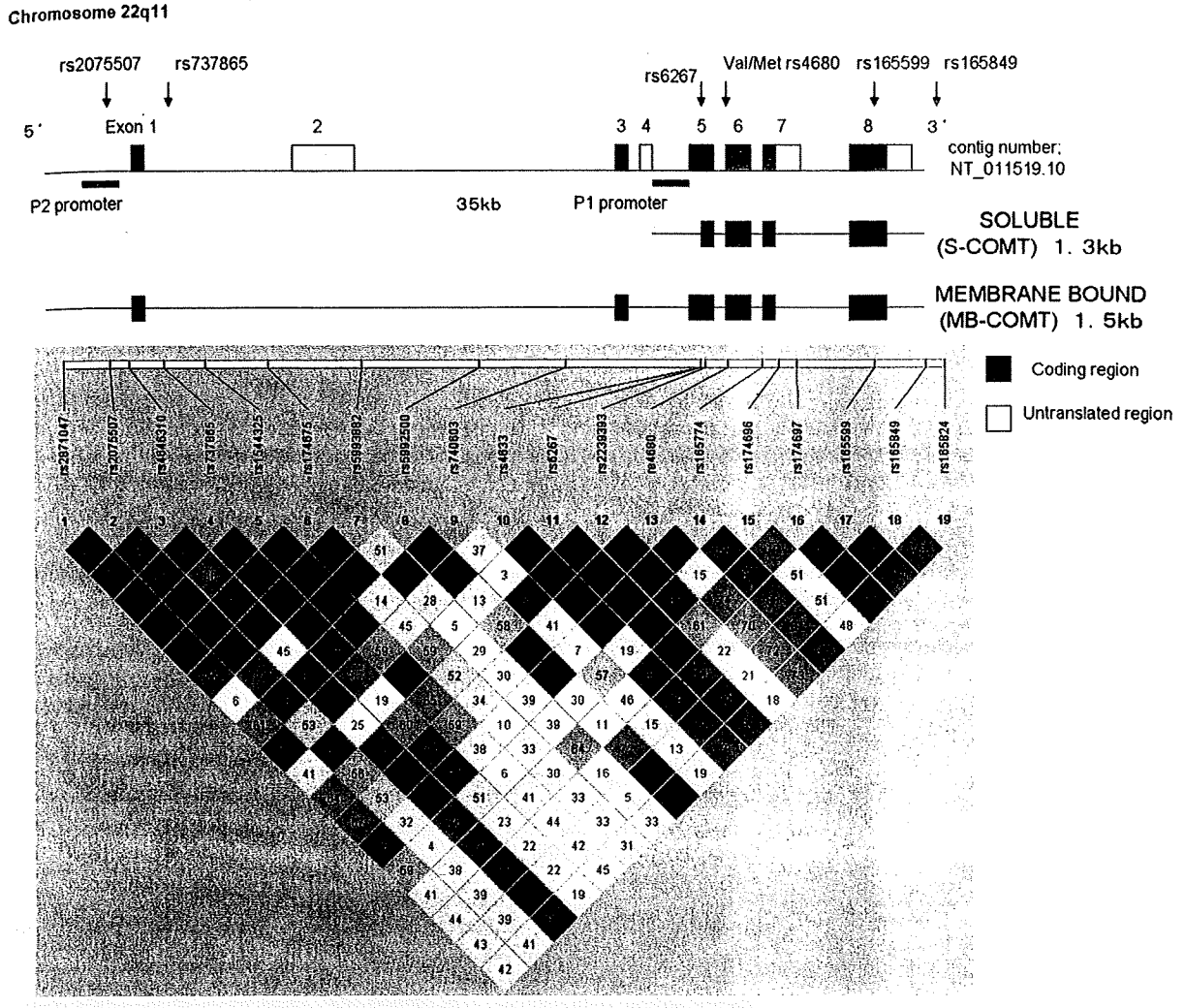

Fig. 1. COMT structure and positions of 'tag SNP' in control sample. LD structure (D') was evaluated with the use of Haploview software.

(rs4680) in *COMT* (Lachman et al., 1996). This is because the Val158/108Met polymorphism has possible functional relevance; the Val allele leads to efficient degradation of dopamine and lower than normal dopamine levels in the brain, since it has higher enzymatic activity than the Met variant (Chen et al., 2004). In addition, recent studies have shown that this possible functional polymorphism (Val/Met) affects executive cognition in the prefrontal cortex (PFC) during working memory (Egan et al., 2001). The above findings suggest that the Val allele may contribute to increased prefrontal dopamine catabolism and impaired PFC function.

A large number of case–control association studies between the Val/Met polymorphism in *COMT* and schizophrenia have been conducted, but the results have been inconsistent; some studies showed a significant association between the Val allele and schizophrenia (Egan et al., 2001; Handoko et al., 2005; Kremer et al., 2003; Li et al., 2000; Shifman et al., 2002; Wonodi et al., 2003), whereas others indicated that the Met allele was associated with schizophrenia (Ohmori et al., 1998; Sazci et al., 2004). The majority, however, found no association (Arinami et al., 2001; Chen et al., 1999; Daniels et al., 1996; Fan et al., 2005; Galderisi et al., 2005; Gallinat et al., 2003; Goghari and Sponheim, 2008; Golimbet et al., 2006; Han et al., 2004, 2006; Herken et al., 2003; Illi et al., 2003; Inada et al., 2003; Iwata et al., 2003; Joo et al., 2005; Joober et al., 2002; Karayiorgou et al., 1998; Kotler et al., 1999; Krabbendam et al., 2006; Liou et al., 2001; Martorell et al., 2008; Muntjewerff et al., 2008; Nicodemus et al., 2007; Numata et al., 2006; Nunokawa et al., 2007; Ohnishi et al., 2006; Park et al., 2002; Poyurovsky et al., 2005; Rujescu et al., 2003; Sanders et al., 2008; Semwal et al., 2002; Strous et al., 1997; Szoke et al., 2006; Thaker et al., 2004; Williams et al., 2005; Yu et al., 2007). In addition, three meta-analyses of this SNP have been reported, but their results were also inconsistent. The first, by Glatt et al. (2003), reported that in case–control studies, especially family-based studies, the Val allele was associated with schizophrenia in the European population. The other two analyses did not provide evidence for a significant association between the Val allele and schizophrenia in either European or Asian populations (Fan et al., 2005; Munafo et al., 2005).

One possible cause of this inconsistency is the existence of an actual causal variant in linkage disequilibrium (LD) with Val/Met polymorphism. Some studies support this, showing a more significant association in haplotypes or other functional

SNPs than from the Val/Met polymorphism alone (Funke et al., 2005; Lee et al., 2005; Sanders et al., 2005; Shifman et al., 2002). For example, (Shifman et al. (2002) reported that haplotypes constructed by three SNPs (Val/Met, rs737865, rs165599) showed the strongest association with schizophrenia in Ashkenazi Jews ($P = 9.5 \times 10^{-8}$).

COMT encodes two transcripts from two promoters in humans (membrane bound; MB-COMT of 1.5 kb from P2/soluble; S-COMT of 1.3 kb from P1: Fig. 1). Funke et al. and Lee et al. also showed that other functional SNPs, rs2075507 (in P2 promoter region of MB-COMT) and rs6267 (change from Alanine to Serine at codon 22/72 in the MB-COMT/S-COMT), were associated with schizophrenia in a European population (Funke et al., 2005) and in a Korean population (Lee et al., 2005).

Since differences in LD among populations may be responsible for the inconsistency of these results, it is important in association studies to select informative genetic variants (e.g. tagging SNPs) that adequately reflect the LD background in the targeted population. Therefore, re-sequencing for mutation screening and LD-based (or gene-based) association studies (Neale and Sham, 2004) is essential to examine the association between COMT and schizophrenia.

In this study, we carried out a systematic mutation scan in the 5′ region and all exon regions and a gene-based case-control study using 19 tagging SNPs in a Japanese sample. We also included an updated meta-analysis for not only Val/Met polymorphism but also other functional SNPs and haplotypes that have been intensively investigated in other studies (rs207055, rs737655, rs6267, rs165599, and rs737865–rs4680–rs165599).

2. Materials and methods

2.1. Mutation scan and case–control study in the Japanese population

2.1.1. Subjects

The subjects in the association analysis were 1118 schizophrenia patients (628 males and 490 females; mean age ± standard deviation; 45.4 ± 15.5 years) and 1100 healthy controls (504 males and 596 females; 38.1 ± 15.2 years). The subjects for the mutation search were 96 patients with schizophrenia. These subjects were also included in the association analysis. All subjects were unrelated to each other and

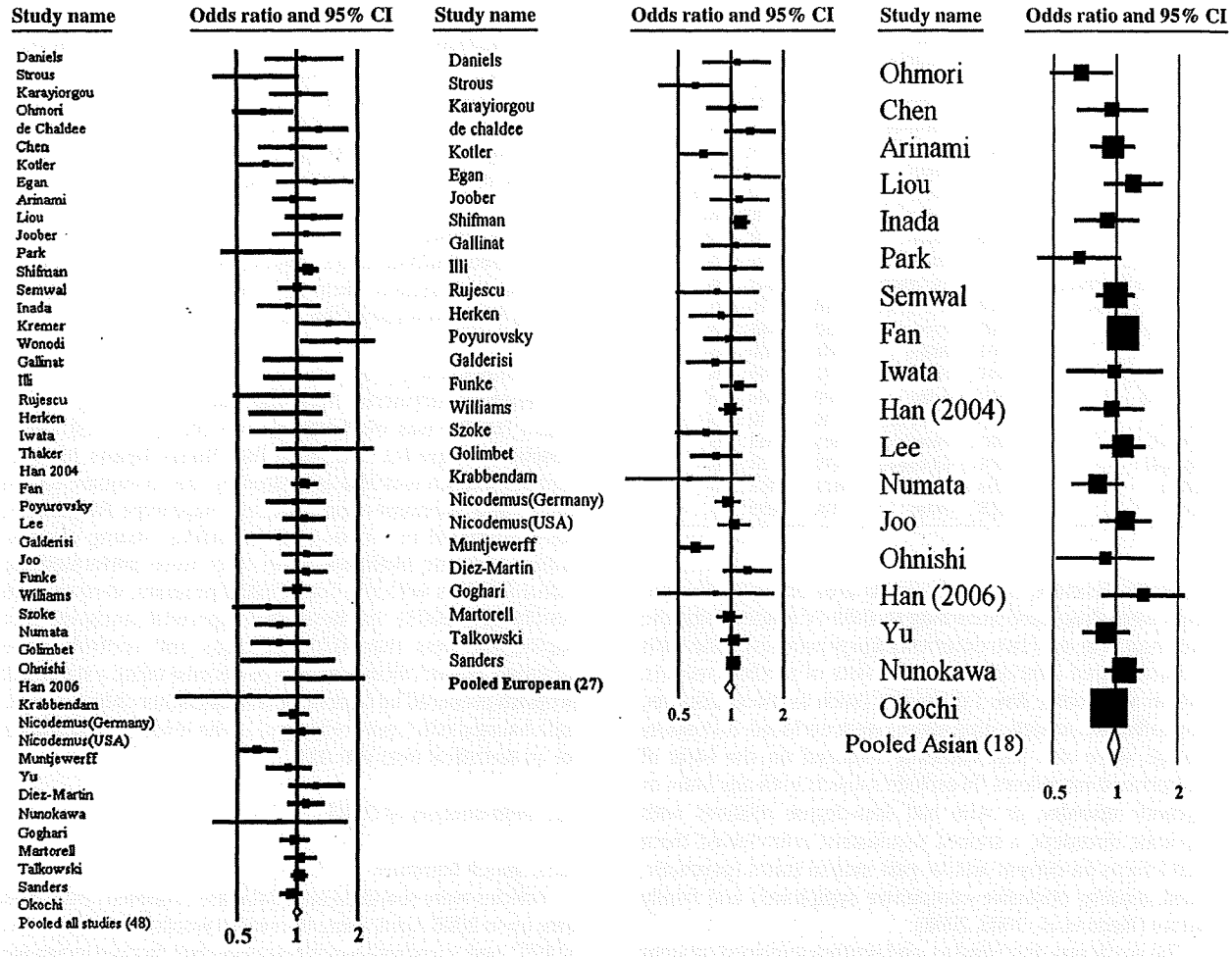

Fig. 2. Forest plots of OR with 95% CI for the Val/Met polymorphism. Results of all pooled studies and subgroup analyses are shown.

Table 1
Studies included in meta-analysis for Val/Met polymorphism.

Author	Year	Ethnic	*n*		Diagnostic system
			Case	CON	
Daniels	1996	European	78	78	III-R
Strous	1997	European	54	87	III-R
Karayiorgou	1998	European	157	129	III
Ohmori	1998	Asian	150	150	IV
de Chaldee	1999	European	136	137	III-R
Chen	1999	Asian	177	99	IV
Kotler	1999	European	92	415	ICD-10
Egan	2001	European	175	55	IV
Arinami	2001	Asian	300	300	III-R
Liou	2001	Asian	198	188	IV
Joober	2002	European	104	96	IV
Park	2002	Asian	103	103	IV
Shifman	2002	European	719	2970	IV
Semwal	2002	Asian	535	262	IV
Inada	2003	Asian	100	201	III-R
Kremer	2003	Other	276	77	III-R
Wonodi	2003	Other	96	79	IV
Gallinat	2003	European	49	170	IV
Illi	2003	European	94	94	IV
Rujescu	2003	European	28	328	IV
Herken	2003	European	143	65	IV
Iwata	2003	Asian	51	69	IV
Thaker	2004	Other	62	53	IV
Han	2004	Asian	168	158	IV
Fan	2005	Asian	862	928	III-R
Poyurovsky	2005	European	113	171	IV
Lee	2005	Asian	320	379	IV
Galderisi	2005	European	111	106	IV
Joo	2005	Asian	239	248	IV
Funke	2005	European	196	467	IV
Williams	2005	European	677	684	IV
Szoke	2006	European	66	50	IV
Numata	2006	Asian	158	317	IV
Golimbet	2006	European	146	130	ICD-10
Ohnishi	2006	Asian	47	76	IV
Han	2006	Asian	132	80	IV
Krabbendam	2006	European	23	21	IV
Nicodemus (Germany)	2007	European	501	627	IV
Nicodemus (USA)	2007	European	296	370	IV
Muntjewerff	2007	European	252	405	IV
Yu	2007	Asian	241	290	IV
Diez-Martin	2007	European	177	141	IV
Nunokawa	2007	Asian	399	440	IV
Goghari	2007	European	39	20	IV
Martorell	2007	European	585	615	IV
Talkowski	2008	European	478	501	IV
Sanders	2008	European	1871	2003	IV
Okochi	2008	Asian	1114	1099	IV

ethnically Japanese, from the central area of Japan. The patients were diagnosed according to DSM-IV criteria with the consensus of at least two experienced psychiatrists on the basis of unstructured interviews and a review of medical records. Patients with other Axis I diagnoses (such as mood disorder, schizoaffective, anxiety, drug abuse) were excluded. All healthy controls were also psychiatrically screened on the basis of unstructured interviews. To exclude subjects with any brain or psychotic disorder, or who had first-degree relatives with psychotic disorders, a trained psychiatrist interviewed them with a focus on current and/or past mental states (psychotic, mood, anxiety, obsessive–compulsive symptoms) and family history (Ikeda et al., 2005, 2008).

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

2.1.2. Mutation scan

Genomic DNA was extracted from peripheral blood of 96 patients with schizophrenia. We amplified the entire exon and P2 promoter regions (promoter of MB-COMT), which are 500 base pairs (bp) upstream from the initial exon. In the human brain, MB-COMT is dominantly detectable. It is important to detect rare variants in the P2 promoter region, which may change MB-COMT expression. Primers for each region were designed with the use of Primer3 software (Whitehead Institute, Cambridge, Massachusetts).

Denaturing high performance liquid chromatography (dHPLC) analysis was carried out to detect mutation. DNA sequencing was then performed using a 3100-Avant Genetic Analyzer (Applied Biosystems, CA). A more detailed description of the methods can be seen in a previous paper (Suzuki et al., 2003) (Supplementary Table 1).

2.1.3. SNP selection and LD evaluation

We first consulted the HapMap database (release#21a, Jan 2007 www.hapmap.org, population: Japanese Tokyo: minor allele frequencies (MAFs) of more than 0.05) and found 48 SNPs covering the COMT gene (5′-flanking regions including 9130 bp from the initial exon and 1559 bp downstream (3′) from the last exon: HapMap database contig number chr22:18300000... 18336530). Then 16 'tagging SNPs' were selected with the criterion of an r^2 threshold greater than 0.8 in 'pair-wise tagging only' mode using the 'Tagger' program (Paul de Bakker, http://www/broad.mit.edu/mpg/tagger), an implement of the HAPLOVIEW software (Barrett et al., 2005).

2.1.4. SNP genotyping

We used TaqMan assays (Applied Biosystems) for all SNPs. Detailed information, including primer sequences, can be seen in Supplementary Table 2.

2.1.5. Statistical analysis

Genotype deviation from the Hardy–Weinberg equilibrium (HWE) was evaluated by the chi-square test (SAS/Genetics, release 8.2, SAS Japan INC, Tokyo, Japan). Marker-trait association was also evaluated by the chi-square test in allele- and genotype-wise analyses. Haplotype frequencies were estimated in a two- to three-marker sliding window fashion and log likelihood ratio tests were performed for global *P*-values with the COCAPHASE program, version 3.0.6 (Dudbridge, 2003). In these haplotype-wise analyses, rare haplotypes (less than 0.05) of cases and controls were excluded. Power calculation was performed using a statistical program prepared by Genetic Power Calculator (http://pngu.mgh.harvard.edu/~purcell//gpc/). The level of significance for all statistical tests was 0.05.

2.2. Meta-analysis of COMT

2.2.1. Search literature

Articles were searched on a database (PubMed) from first date up to 2008 April, using the search words 'schizophrenia', 'COMT', and 'catechol-O-methyltransferase'. In cases when we could not obtain detailed information about allele or genotype

Table 2
Association analysis of tagging SNPs in COMT.

Gene	Marker IDs		Distance to next SNP (bp)	N[a]		MAF[b]		M/M[c]		M/m[d]		m/m[e]		*P*-values		Haplotype			
				SCZ	CON	SCZ	CON	SCZ	CON	SCZ	CON	SCZ	CON	genotype	allele	2 window (*P*-values)		3 window (*P*-values)	
COMT	M1	rs2871047	0	1112	1093	0.344	0.35	492	471	473	478	147	144	0.838	0.699				
	M2	rs2075507	1761	1067	1096	0.281	0.287	559	554	415	453	93	89	0.5	0.649	0.957		0.953	
	M3	rs4646310	714	1106	1097	0.065	0.072	969	944	129	148	8	5	0.319	0.396		0.717		0.886
	M4	rs737865	1315	1106	1087	0.255	0.258	625	600	396	411	85	76	0.569	0.814	0.712		0.967	0.902
	M5	rs1544325	1547	1115	1089	0.3	0.298	551	533	458	462	106	94	0.694	0.884		0.919		0.969
	M6	rs174675	2383	1110	1084	0.446	0.452	343	331	543	525	224	228	0.884	0.684	0.945		0.992	0.965
	M7	rs5993882	3482	1113	1101	0.123	0.125	861	840	229	246	23	15	0.288	0.855		0.882		0.895
	M8	rs5992500	4414	1116	1097	0.049	0.055	1008	982	105	109	3	6	0.534	0.418	0.975		0.58	0.811
	M9	rs740603	3230	1114	1096	0.379	0.383	443	420	497	511	174	165	0.637	0.763		0.824		0.612
	M10	rs4633	5058	1116	1100	0.321	0.307	527	536	460	451	129	113	0.574	0.317	0.811		0.551	0.625
	M11	rs6267	28	1118	1100	0.087	0.086	933	915	175	180	10	5	0.413	0.92		0.561		0.22
	M12	rs2239393	165	1111	1087	0.264	0.258	612	603	410	407	89	77	0.71	0.619	0.91		0.259	0.481
	M13	rs4680	843	1114	1099	0.341	0.325	497	499	474	485	143	115	0.215	0.264		0.38		0.109
	M14	rs165774	1290	1115	1091	0.174	0.164	765	759	311	304	39	28	0.438	0.403	0.547			
	M15	rs174696	615	1112	1088	0.429	0.424	357	367	555	518	200	203	0.555	0.748		0.779		
	M16	rs174697	656	1111	1097	0.368	0.391	446	408	511	520	154	169	0.304	0.123	0.156			
	M17	rs165599	2949	1098	1089	0.432	0.457	367	328	512	526	219	235	0.234	0.1		0.305		
	M18	rs165849	1888	1118	1085	0.439	0.461	359	318	536	532	223	235	0.313	0.132	0.119			
	M19	rs165824	697	1104	1097	0.413	0.434	389	357	517	527	198	213	0.369	0.161		0.11		

[a] *N* = number, SCZ = schizophrenia, CON = control.
[b] MAF = minor allele frequency.
[c] M/M = major allele/major allele.
[d] M/m = major allele/minor allele.
[e] m/m = minor allele/minor allele.

or haplotype frequencies in the article, we tried to contact the author directly or we referred to the 'SzGene database' (http://www.schizophreniaforum.org/res/sczgene/default.asp) (Allen et al., 2008) (however, we could not obtain results on haplotype frequencies in the study by Shifman et al. (2002)).

2.2.2. Criteria for inclusion

From the database search, we selected population-based case–control studies that investigated the genotype and allele frequencies of the Val/Met polymorphism or other *COMT* polymorphisms (in patients diagnosed according to the ICD or DSM criteria and healthy controls). Duplication articles were excluded. Studies in which control allele frequencies deviated from HWE ($P<0.01$) were also excluded.

A total of 48 population-based studies (Arinami et al., 2001; Chen et al., 1999; Daniels et al., 1996; de Chaldee et al., 1999; Diez-Martin et al., 2007; Egan et al., 2001; Fan et al., 2005; Funke et al., 2005; Galderisi et al., 2005; Gallinat et al., 2003; Goghari and Sponheim, 2008; Golimbet et al., 2006; Han et al., 2004, 2006; Herken et al., 2003; Illi et al., 2003; Inada et al., 2003; Iwata et al., 2003; Joo et al., 2005; Joober et al., 2002; Karayiorgou et al., 1998; Kotler et al., 1999; Krabbendam et al., 2006; Kremer et al., 2003; Lee et al., 2005; Liou et al., 2001; Martorell et al., 2008; Muntjewerff et al., 2008; Nicodemus et al., 2007; Numata et al., 2006; Nunokawa et al., 2007; Ohmori et al., 1998; Ohnishi et al., 2006; Park et al., 2002; Poyurovsky et al., 2005; Rujescu et al., 2003; Sanders et al., 2008; Semwal et al., 2002; Shifman et al., 2002; Strous et al., 1997; Szoke et al., 2006; Talkowski et al., 2008; Thaker et al., 2004; Williams et al., 2005; Wonodi et al., 2003; Yu et al., 2007) were identified using our search criteria for this meta-analysis (including our case-control study using a Japanese population) (Table 1).

Table 3
Association analysis of attractive haplotypes from other studies global *P*-values were obtained by the COCAPHASE program rare haplotypes (less than 0.05) were excluded.

Haplotype	Global *P*-values
rs4680–rs165599	0.396
rs737865–rs4680	0.206
rs6267–rs4680	0.529
rs4633–rs4680	0.274
rs4680–rs165849	0.166
rs737865–rs4680–rs165599	0.301
rs4680–rs165599–rs165849	0.151
rs2075507–rs737865–rs4680–rs165599	0.193
rs737865–rs4633–rs4680–rs165599	0.242
rs737865–rs6267–rs4680–rs165599	0,738

Table 4
Results of meta-analysis for Val/Met polymorphism in COMT.

	OR (95% CI)	*P*-value (*Z*)	*P*-value (*Q*)
Val/Met (rs4680)			
G/A			
All studies (48)[a]	0.989 (0.942–1.039)	0.667	0.011
European (27)	0.971 (0.909–1.038)	0.392	0.016
Asian (18)	0.984 (0.924–1.048)	0.62	0.378
(GG + GA)/AA			
All studies (48)	0.992 (0.932–1.056)	0.804	0.807
European (27)	0.988 (0.919–1.062)	0.747	0.525
Asian (18)	0.918 (0.81–1.04)	0.677	0.478
GG/(GA + AA)			
All studies (48)	1.020 (0.952–1.092)	0.575	0.043
European (27)	1.030 (0.943–1.124)	0.511	0.192
Asian (18)	0.976 (0.881–1.082)	0.65	0.107

COMT: Catechol-o-methyltransferase, OR: odds ratio, CI: confidence interval. *P*-value (*Z*): the significance of the pooled OR was determined using a *Z*-test. *P*-value (*Q*): the heterogeneity was checked using a *Q* statistic test.
[a] (): the number of studies.

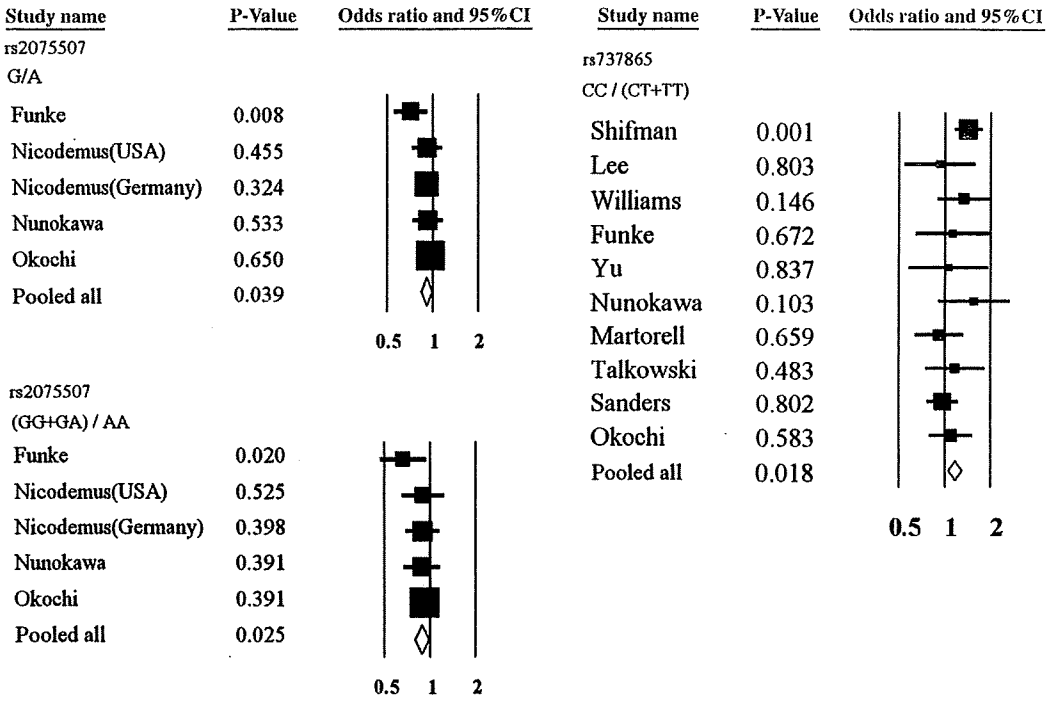

Fig. 3. Forest plots of OR with 95% CI for the rs2075507 (G/A, GG + GA/AA) and rs737865 (CC/CT + TT). Marginal associations were detected in each SNP.

2.2.3. Statistical analyses

A two-by-two table in which subjects were classified by diagnosis and type of allele (additive, dominant and recessive models) was constructed. Random-effect models were adopted to check the heterogeneity using a Q statistic test in the combined studies. Odds ratios (ORs) were pooled using DerSimonian and Laird methods. The significance of the pooled OR was determined using a Z-test. Publication bias was assessed using a funnel plot asymmetry with Egger's test. The statistical significance was set at 0.05. All data were analyzed using Comprehensive Meta Analysis (Version 2.0).

To correct for problems of multiple comparisons, we applied the Benjamini–Hochberg (BH) method, which is a procedure to control for false discovery rate (FDR) (Benjamini and Hochberg, 2000).

3. Result

3.1. Mutation scan and case–control study in the Japanese population

Our mutation scan detected a number of SNPs in this population that are listed in the dbSNP database (rs2020917, rs4633, rs6267, rs4818, rs4680, rs4646316 and rs165774), but did not find any novel SNPs. All SNPs detected in this mutation scan were unlikely to have functional relevance, since they are synonymous or in the branch site regions. We then performed a genetic case–control study using the tagging SNPs from the HapMap database and possible functional SNPs. Genotype frequencies of subjects and controls did not deviate significantly from HWE (Table 2). There was no significant association in the allele/genotype-wise analysis or in the haplotype analysis (Tables 2 and 3). We obtained power of more than 80% for the detection of association when we set the genotype relative risk at 1.19–1.57 under a multiplicative model of inheritance.

3.2. Meta-analysis

3.2.1. Val108/158Met polymorphism (rs4680)

All population-based studies that were identified by our search criteria included an analysis for Val/Met polymorphism (total sample sizes were 13088 cases and 16531 controls). No association of this SNP to schizophrenia was found in the allele frequencies (G/A: pooled OR = 0.989, CI = 0.942–1.039, $P(Z) = 0.667$, GG + GA/AA: pooled OR = 0.992, CI = 0.932–1.056, $P(Z) = 0.804$, GG/GA + AA: pooled OR = 1.020, CI = 0.952–1.092, $P(Z) = 0.575$; Table 4).

Heterogeneity among these studies was detected under some models (G/A: $P(Q) = 0.011$, GG/GA + AA: $P(Q) = 0.043$). We therefore divided samples into two groups in accordance with populations: European (27 population-based studies) and Asian (18 population-based studies). In this subgroup analyses, no association was detected from either European or Asian samples (Table 4, Fig. 2), but caution is needed because heterogeneity still existed in the European samples (G/A: $P(Q) = 0.016$ for Europeans).

No publication bias was detected for this SNP (Supplementary Fig. 1).

3.2.2. Other SNPs (rs2075507, rs737865, rs6267, rs165599) and haplotypes

For rs2075507, 5 population-based studies (Funke et al., 2005; Nicodemus et al., 2007; Nunokawa et al., 2007) were

Table 5
Results of meta-analysis for 4 functional SNPs and haplotype in COMT.

	OR (95% CI)	*P*-value (*Z*)	*P*-value (*Q*)
rs2075507			
G/A (5)[a]	0.912 (0.835–0.995)	**0.039**	0.341
(GG+GA)/AA	0.882 (0.79–0.984)	**0.025**	0.559
GG/AG+AA	0.92 (0.776–1.090)	0.333	0.421
rs737865			
C/T (10)	1.041 (0.978–1.108)	0.207	0.184
(CC+CT)/TT	1.014 (0.946–1.088)	0.691	0.374
CC/(CT+TT)	1.155 (1.025–1.303)	**0.018**	0.336
rs6267			
T/G (3)	0.781 (0.564–1.081)	0.136	0.031
(TT+TG)/GG	0.765 (0.53–1.103)	0.152	0.021
TT/(GT+GG)	1.49 (0.452–4.913)	0.512	0.176
rs165599			
G/A (11)	1.032 (0.960–1.108)	0.396	0.018
(GG+GA)/AA	1.016 (0.940–1.098)	0.691	0.207
GG/(GA+AA)	1.076 (0.940–1.232)	0.286	0.033
rs737865–rs4680–rs165599			
Haplotype analysis (4)			
CGG[b]	0.904 (0.763–1.071)	0.244	0.456
TGA[c]	0.992 (0.876–1.123)	0.895	0.261
TGG[d]	1.063 (0.942–1.201)	0.321	0.686

COMT: Catechol-o-methyltransferase, OR: odds ratio, CI: confidence interval.
P-value (*Z*): The significance of the pooled OR was determined using a *Z*-test.
P-value (*Q*): The heterogeneity was checked using a *Q* statistic test.
Bold numbers represent significant *P*-value (<0.05).
[a] (): the number of studies.
[b] Shifman et al. reported.
[c] J. Chen et al. reported.
[d] Handoko et al. reported.

identified (total sample sizes: 2456 cases and 3000 controls) and there was no evidence for heterogeneity. We found a trend for association between allele frequencies and schizophrenia (G/A: pooled OR = 0.912, CI = 0.835–0.995, $P(Z) = 0.039$, GG + GA/AA: pooled OR = 0.882, CI = 0.79–0.984, $P(Z) = 0.025$; Table 5).

For rs737865, 10 population-based studies (Funke et al., 2005; Lee et al., 2005; Martorell et al., 2008; Nunokawa et al., 2007; Sanders et al., 2008; Shifman et al., 2002; Talkowski et al., 2008; Williams et al., 2005; Yu et al., 2007) were identified (total sample sizes: 6599 cases and 9323 controls) and no heterogeneity was found. Again, a trend for association was detected (CC/CT + TT: pooled OR = 1.155, CI = 1.025–1.333, $P(Z) = 0.018$).

However, two other SNPs (rs6267, rs165599) and haplotype analyses did not find evidence of a significant association (Table 5, Fig. 3).

With further checking of the aforementioned marginal associations in rs2075507 and rs737865, after correcting for multiple testing by FDR, both *P*-values from these SNPs were found to be larger than the *Q*-value, indicating the significance for these SNPs was derived from type I error (rs2075507 G/A $P = 0.039 > Q = 0.0062$, GG + GA/AA $P = 0.025 > Q = 0.0041$, rs737865 CC/CT + TT $P = 0.018 > Q = 0.002$).

No publication bias was found for any of the SNPs.

4. Discussion

4.1. LD-based-association analysis of COMT in the Japanese population

In this LD-based case–control study, our data did not show sufficient evidence for an association between possible functional SNPs or tagging SNPs and schizophrenia in the Japanese population. The LD-based strategy we adopted was the minimum required to examine the association of *COMT* with schizophrenia, considering a recent study by Mukherjee et al. (2008) which showed that several haplotypes, but not SNPs by themselves such as Val/Met polymorphism, may be associated with schizophrenia because of LD differences among populations. In fact, they found that haplotype frequencies differed even among European populations in some regions of *COMT*. Therefore, to contain sufficient information and be cost-effective, variants used in association studies should be, at minimum, selected based on information from the HapMap database as appropriate tagging SNPs. Samples should also be from homogeneous population settings. In addition, mutation scans are important in order to detect rare but functional variants in all of COMT. This is partly because it is likely that such rare variants do not overlap among all populations (Pritchard, 2001).

Our results showed a clear lack of association of several tagging SNPs in *COMT* with schizophrenia in the Japanese population. In an explorative analysis, we examined the association of a large number of haplotypes that were constructed by all combinations of SNPs; however, no association was detected (the minimum *P*-value = 0.151, haplotype rs4680–rs165599–rs165849). Our sample size was one of the largest among genetic association studies for *COMT* to avoid overlooking false negative results, and therefore the results of the analysis should be reliable.

Some limitations should be noted with regard to interpretation of our results. Firstly, we scanned only P2 promoter and exon regions in this mutation scan. We should also note the possibility that far genomic regions or introns affect the gene's expression or splicing patterns. Secondly, our samples were not assessed by standard structured interviews, increasing the chance of false negatives due to misdiagnosis or sampling bias.

4.2. Updated meta-analyses of attractive SNPs and haplotypes

To date, three independent groups have reported meta-analyses of Val/Met polymorphism in *COMT* with schizophrenia; one showed a significant association but the other two showed no evidence for association. Our results indicate that the Val/Met polymorphism and four other functional SNPs may not play a major role in schizophrenia. However, considerable heterogeneity for Val/Met polymorphism was detected among all population-based studies. Therefore, we performed the following subgroup analysis by population, but heterogeneity still existed among the European studies. Heterogeneity is considered to be partly due to population stratification by LD differences and sampling bias.

It is clear that LD differences make interpretation from the results of meta-analysis difficult; positive results may be derived from false positives due to population stratification,

whereas negative results may be induced by simply overlooking population-specific effects of the examined variants. To overcome these difficulties in future meta-analysis, comparison of gene-wide significance from populations considered to have LD differences, adequate sample size, and accurate phenotype and diagnosis definition will be needed (Moskvina et al., 2009).

In conclusion, our results suggest that COMT may not play a major role in schizophrenia. However, there are reported associations of endophenotype with schizophrenia. A recent report showed a meta-analysis of WCST in schizophrenia and controls (Barnett et al., 2007). Further studies will be needed to examine the association between COMT and PFC function.

Role of funding source

This work was supported in part by research grants from the Ministry of Education, Culture, Sports, Science and Technology, the Ministry of Health, Labor and Welfare, and the Japan Health Sciences Foundation (Research on Health Sciences Focusing on Drug Innovation).

Contributors

All authors contributed to and have approved the final manuscript.

Conflict of interest

None.

Acknowledgements

We thank Ms S Nakaguchi and Ms M Miyata for their technical support.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.schres.2009.02.019.

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Eur Arch Psychiatry Clin Neurosci (2009) 259:293–297 DOI 10.1007/s00406-009-0869-4

ORIGINAL PAPER

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Association study of clock gene (*CLOCK*) and schizophrenia and mood disorders in the Japanese population

Received: 2 September 2008 / Accepted: 13 January 2009 / Published online: 17 February 2009

Abstract Recently the clock genes have been reported to play some roles in neural transmitter systems, including the dopamine system, as well as to regulate circadian rhythms. Abnormalities in both of these mechanisms are thought to be involved in the pathophysiology of major mental illness such as schizophrenia and mood disorders including bipolar disorder (BP) and major depressive disorder (MDD). Recent genetic studies have reported that *CLOCK*, one of the clock genes, is associated with these psychiatric disorders. Therefore, we investigated the association between the six tagging SNPs in *CLOCK* and the risk of these psychiatric disorders in Japanese patients diagnosed with schizophrenia (733 patients), BP (149) and MDD (324), plus 795 Japanese controls. Only one association, with schizophrenia in females, was detected in the haplotype analysis ($P = 0.0362$). However, this significance did not remain after Bonferroni correction ($P = 0.0724$). No significant association was found with BP and MDD. In conclusion, we suggest that *CLOCK* may not play a major role in the pathophysiology of Japanese schizophrenia, BP and MDD patients. However, it will be important to replicate and confirm these findings in other independent studies using large samples.

Key words schizophrenia · bipolar disorder · major depressive disorder · *CLOCK* · tagging SNP

Electronic supplementary material: The online version of this article (doi:10.1007/s00406-009-0869-4) contains supplementary material, which is available to authorized users.

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Introduction

Sleep disturbances are commonly observed in psychiatric disorders, and sleep manipulations can influence clinical status. Abnormalities in circadian rhythms have been reported to be involved in the pathophysiology of major mental illness such as schizophrenia and mood disorders [2, 24, 26, 27]. Also, because all psychotropic drugs have actions on the systems of neurotransmitters such as dopamine and serotonin in the brain, altered neural transmission is hypothesized to be a susceptibility factor for major mental illness [21, 29]. Recently these neurotransmitter systems have been reported to have reciprocal interactions with circadian rhythms [3, 40].

Clock genes were also discussed to regulate not only circadian rhythms but dopamine neural transmission [25]. Recently, *Per2*, one of the circadian clock genes, was shown to alter dopamine levels in the caudate putamen and the nucleus accumbens, mediated by reduced expression and activity of monoamine oxidase A, and its mutant mice showed

behaviors that resembled human mood disorders [16]. Abnormalities in dopamine neural transmission are known to be involved in the pathophysiology of schizophrenia [19], bipolar disorder (BP) [7] and major depressive disorder (MDD) [31]. A recent study has reported that plasma cortisol levels are elevated in schizophrenia and BP patients compared with controls [14]. Adrenal steroid hormones levels change based on circadian rhythms, it has been suggested and this mechanism may be involved in the development of insomnia and psychiatric disorders [10]. In addition, some genetic studies showed significant associations between schizophrenia/schizo-affective disorder and timeless homolog gene (*TIMELESS*) or period homolog 3 gene (*PER3*), between BP and Bmal1 gene (*ARNTL*) *or TIMELESS* or *PER3* [22, 32]. These facts suggest a crucial relationship between circadian rhythms and psychiatric disorders, and so genes associated with the molecular clock mechanism are good candidates for the etiology of psychiatric disorders. We thought these psychiatric disorders may have some shared mechanisms as to circadian rhythms and considered that it was reasonable to assess all these disorders.

Recent genetic studies showed significant associations of a SNP (T3111C: rs1801260) in *CLOCK*, one of the clock genes, with Japanese schizophrenia [39] and clinical features of BP such as a high recurrence rate [5, 6, 35]. In an animal study using *CLOCK* mutant mice that showed mania-like behavior, this behavior was reversed by lithium treatment [34]. In addition, *CLOCK* mutant mice showed altered regulation of dopamine release in the ventral tegmental area mediated tyrosine hydroxylase regulated by circadian rhythms [28, 41]. Therefore, *CLOCK* would seem to be a good candidate gene for the pathophysiology of psychiatric disorders.

The *CLOCK* gene (OMIM *601851, 25 exons in this genomic region spanning 115.138 kb) is located on 4q12. This genomic region was shown to be closely related to susceptibility for schizophrenia [17, 38], BP [8, 15, 23] and MDD [11, 12]. Therefore, in this study, we aim to examine the genetic association between *CLOCK* and schizophrenia, BP and MDD in the Japanese population. To do this, we applied the recently recommended strategy of 'gene-based' association analysis [30]. We conducted a case–control association analysis using relatively large samples by selecting 'tagging SNPs' from the HapMap database.

Materials and methods

Subjects

The subjects in the association analysis were 733 schizophrenia patients [393 males and 340 females; mean age ± standard deviation (SD) 36.3 ± 18.4 years], 149 with BP (79 males and 70 females: 95 patients with bipolar I disorder and 54 patients with bipolar II disorder; 47.8 ± 14.6 years), 324 with MDD (159 males and 165 females; 47.5 ± 16.1 years) and 795 healthy controls (347 males and 448 females; 37.6 ± 14.3 years). Patients were grouped according to the following DSM-IV subtypes of schizophrenia: Paranoid Type (n = 216), Disorganized Type (n = 221), Catatonic Type (n = 29), Residual Type (n = 142), Undifferentiated Type (n = 125). The patients were diagnosed according to DSM-IV criteria with the consensus of at least two experienced psychiatrists on the basis of unstructured interviews and a review of medical records. All healthy control subjects were also psychiatrically screened based on unstructured interviews. None had severe medical complications such as cirrhosis, renal failure, heart failure or other Axis-I disorders according to DSM-IV. No structured methods were used to assess psychiatric symptoms in the controls, who included hospital staff, their families and medical students. None of the subjects were known to be related to each other, and all were ethnically Japanese. Written informed consent was obtained from each subject. This study was approved by the ethics committees at Fujita Health University and Nagoya University Graduate School of Medicine.

SNP selection and linkage disequilibrium evaluation

We first consulted the HapMap database (release#23.a.phase2, Mar 2008, http://www.hapmap.org, population: Japanese Tokyo: minor allele frequencies (MAFs) of more than 0.1) and included 106 SNPs covering *CLOCK* (5′-flanking regions including about 2 kb from the initial exon and about 5 kb downstream (3′) from the last exon: HapMap database contig number chr4: 55990340.. 56108588). Then six 'tagging (tag) SNPs' including rs1801260: T3111C (called SNP5 in this study) associated with Japanese schizophrenia [39] were selected with the criteria of an r^2 threshold greater than 0.8 in 'pairwise tagging only' mode using the 'Tagger' program (Paul de Bakker, http://www/broad.mit.edu/mpg/tagger) of the HAPLOVIEW software [4], in the following association analysis.

SNP genotyping

We used TaqMan assays (Applied Biosystems, Foster City, CA, USA) for all SNPs. Detailed information is available on request.

Statistical analysis

Genotype deviation from the Hardy–Weinberg equilibrium (HWE) was evaluated by chi-square test (SAS/Genetics, release 8.2, SAS Japan Inc., Tokyo, Japan). Marker-trait association analysis was used to evaluate allele- and genotype-wise association with the chi-square test (SAS/Genetics, release 8.2, SAS Japan Inc., Tokyo, Japan), and haplotype-wise association analysis was evaluated by a likelihood ratio test using the COCAPHASE2.403 program [13]. Bonferroni's correction was used to control inflation of the type I error rate. Power calculation was performed using genetic power calculator [33]. The significance level for statistical tests was 0.05.

Results

Genotype frequencies were in HWE for this SNP. Linkage disequilibrium structures from the HapMap database can be seen in Fig. 1. The LD structures of schizophrenia, BP, MDD and control samples were almost the same (Fig. 1). In addition, LD from SNP1 to SNP4 was very tight in our control samples (r^2 more than 0.9), although we selected tag SNPs from HapMap database with the criteria of r^2 more than

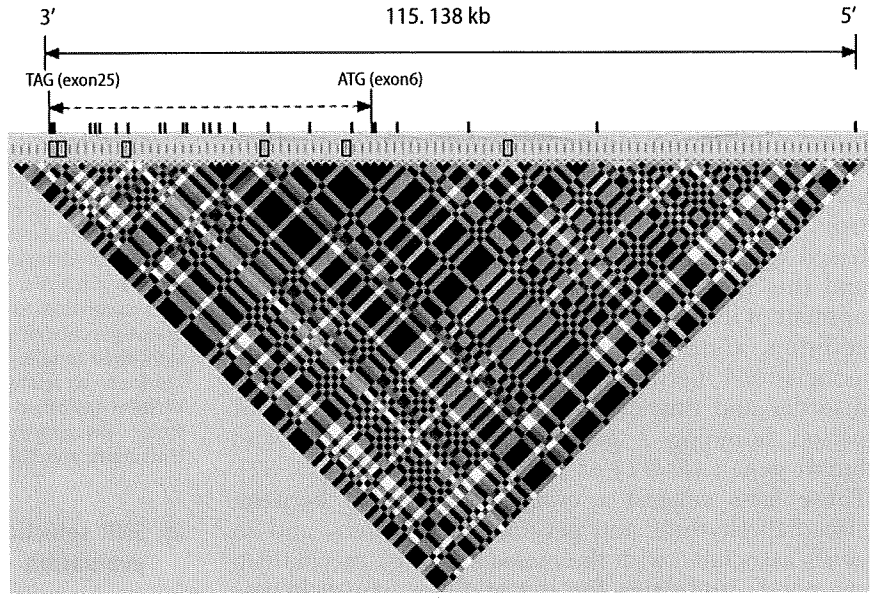

Fig. 1 LD evaluation and tagging SNPs in *CLOCK*. *Black bars* represent exons of *CLOCK*. Tagging SNPs selected from HapMap database are represented by *black boxes*. The *color* scheme is based on r^2 value. LD structure of *CLOCK* is very tight and roughly one block. The *color* scheme is based on r^2 value. Other information can be seen at the HAPLOVIEW website

Table 1 Association analysis of tagging SNPs in CLOCK

SNP ID[a]		Phenotype	MAF	*n*	Genotype distribution			*P* value		
					M/M	M/m	m/m	HWE	Genotype	Allele
SNP1	rs11939815	Control	0.216	795	490	266	39	0.708		
	G > T	SCZ	0.207	733	460	243	30	0.767	0.513	0.724
		MDD	0.221	324	192	121	11	0.123	0.822	0.302
		BP	0.228	149	88	54	7	0.724	0.694	0.406
SNP2	rs11931061	Control	0.221	795	485	268	42	0.532		
	A > G	SCZ	0.212	733	454	247	32	0.827	0.536	0.699
		MDD	0.224	324	190	123	11	0.0948	0.902	0.208
		BP	0.228	149	88	54	7	0.724	0.795	0.821
SNP3	rs11133385	Control	0.220	795	485	270	40	0.760		
	A > G	SCZ	0.222	733	452	236	45	0.0615	0.881	0.541
		MDD	0.221	324	193	119	12	0.222	0.977	0.482
		BP	0.235	149	88	52	9	0.723	0.574	0.837
SNP4	rs3736544	Control	0.220	795	487	266	42	0.472		
	G > A	SCZ	0.205	733	460	246	27	0.402	0.296	0.317
		MDD	0.222	324	192	120	12	0.199	0.914	0.334
		BP	0.232	149	88	53	8	0.996	0.663	0.875
SNP5	rs1801260	Control	0.161	795	563	208	24	0.373		
	T > C	SCZ	0.148	733	532	185	16	0.986	0.321	0.519
		MDD	0.157	324	231	84	9	0.684	0.833	0.972
		BP	0.158	149	106	39	4	0.856	0.887	0.976
SNP6	rs3749474	Control	0.380	795	311	364	120	0.427		
	T > C	SCZ	0.359	733	301	338	94	0.953	0.228	0.412
		MDD	0.356	324	119	160	45	0.450	0.793	0.547
		BP	0.376	149	58	70	21	0.987	0.895	0.940

[a]Major allele > minor allele
SCZ schizophrenia, *MDD* major depressive disorder, *BP* bipolar disorder, *MAF* minor allele frequency, *M* major allele, *m* minor allele, *HWE* Hardy–Weinberg equilibrium

0.8. We did not find an association between these tag SNPs and Japanese schizophrenia, BP or MDD in any of the analyses (Tables 1, 2). It is known that there are sex differences in not only the pathophysiology of schizophrenia [18] but also in circadian rhythms [20], and we detected slight gender differences in LD structures constructed of tag SNPs of both schizophrenia samples in this study (Supplementary Figure 1). To further investigation of these associations, we performed an explorative single marker and haplotype-wise analysis of subjects divided by sex. Only one association was detected, with schizophrenia females, in the haplotype-wise analysis ($P = 0.0362$) (Supplementary Table 4). However, this significance did not remain after Bonferroni correction ($P = 0.0724$) (Supplementary Table 4). Also, no association was detected in either sex in MDD or BP (Supplementary Tables 1, 2, 3, 4). In the power analysis, we

Table 2 Haplotype-wise analysis of tagging SNPs in *CLOCK*

Haplotype	Phenotype	Individual haplotype frequency	Individual *P* value	Phenotype	Global *P* value
GAAGTT	Control	0.625			
	SCZ	0.651	0.190	SCZ	0.340
	MDD	0.617	0.672	MDD	0.883
	BP	0.629	0.948	BP	0.957
GAAGCC	Control	0.158			
	SCZ	0.139	0.202		
	MDD	0.165	0.648		
	BP	0.150	0.782		
TGGATC	Control	0.217			
	SCZ	0.210	0.671		
	MDD	0.218	0.926		
	BP	0.220	0.867		

SCZ schizophrenia, *MDD* major depressive disorder, *BP* bipolar disorder

obtained more than 80% power for the detection of association when we set the genotype relative risk for *CLOCK* at 1.25–1.52 in schizophrenia, 1.76–1.85 in BP and 1.58–1.95 in MDD, under a multiplicative model of disease risk.

Discussion

In this study, only one association with schizophrenia in females was detected in the haplotype analysis ($P = 0.0362$), but this significance did not remain after Bonferroni correction ($P = 0.0724$). Also, we could not replicate the association between SNP5 (rs1801260: T3111C) and schizophrenia found in an earlier study [39], using larger Japanese schizophrenia and control samples. At this SNP, Takao et al. [39] showed higher MAFs of schizophrenia (MAFs: 0.224) compared with those of controls (MAFs: 0.141), although our study did not detect a significant difference with MAFs in schizophrenia or any specific gender subgroup compared with control. Also, there has been opened MAFs: 0.198 in Japanese HapMap database. In addition, LD from SNP1 to SNP4 was very tight in our control samples (r^2 more than 0.9), despite our selection of tag SNPs from HapMap database with the criteria of r^2 more than 0.8 (Minimum r^2 from SNP1 to SNP4 was 0.754 according to the database). So the differences of MAFs and r^2 in the Takao's study and the HapMap database with this study might be influenced by the sample size of each studies [39].

Similar to our study, several other investigations have found no association between *CLOCK* and BP or MDD using case-control samples and family based samples [1, 22, 32, 35, 36].

A few points of caution should be noted in interpreting our results. First, the lack of association may be due to biased samples, such as small sample sizes, especially BP and MDD samples or unmatched age- or gender-samples. Because our BP and MDD samples are small, there are possibilities of typeIIerrors in the results of association analysis for mood disorders statistically. Also, although we included subgroup analyses divided by gender, careful interpretation is needed with respect to the association of schizophrenia itself. On average, the controls are much younger than the patients. This means that a number of young controls may go on to develop one these disorders, most likely MDD, since the incidence of major depression is as high as 5% or more. Our subjects did not undergo structured interviews. MDD patients who are not diagnosed by structured interview may develop BP in the future [9, 37]. In addition, female schizophrenia has possibility to develop in the menopause. However, in this study patients were carefully diagnosed according to DSM-IV criteria with consensus of at least two experienced psychiatrists on the basis of a review of medical records. In addition, when we found a misdiagnosis of a patient, we promptly excluded the misdiagnosed case in consideration of the precision of our sample. Second, we did not include a mutation scan to detect rare variants with functional effects. However, it is difficult to evaluate the association of such extremely rare variants (e.g. MAFs < 0.01) from the viewpoint of power.

In conclusion, we suggest that *CLOCK* may not play a major role in the pathophysiology of schizophrenia, BP and MDD in the Japanese population. However, it will be important to replicate and confirm these findings in other independent studies using larger samples.

Acknowledgments We thank Ms. M. Miyata and Ms. S. Ishihara for their technical support. This work was supported in part by research grants from the Japan Ministry of Education, Culture, Sports, Science and Technology, the Ministry of Health, Labor and Welfare, and the Health Sciences Foundation (Research on Health Sciences focusing on Drug Innovation).

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