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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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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 $\pm$ standard deviation (SD), 42.6 $\pm$ 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 $\pm$ SD, 39.5 $\pm$ 14.7 years). A total of 351 healthy volunteers (193 male and 158 female; mean age $\pm$ SD, 33.5 $\pm$ 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.,

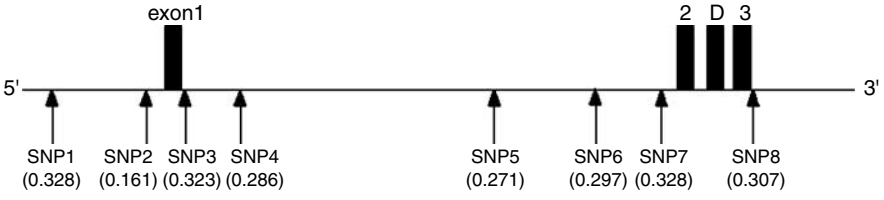

Figure 1 Genomic structure of HTR7 with SNPs used in LD mapping. Exon D is an alternative splice exon. Numbers in parentheses represent minor allele frequencies (MAFs) of 96 controls.

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 (C11756841)	12040	TaqMan				
SNP5 (G>A) rs12412496 (C416286)	56980	TaqMan				
SNP6 (A>C) rs2420367 (C11756716)	19982	PCR-RFLP	TTTTCCATCCCACTTTCAGC	ACAGGCAGAAAACAGCGAAC		*Sml*I
SNP7 (C>T) rs4933194 (C375195)	14816	TaqMan				
SNP8 (A>G) rs1045949	10643	Direct sequence	AGGGCACTGTCCTTTCAGAC	ATGTCTGCCCTTCAGCCATT		

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 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

					Genotypic distribution[b]							Allelic distribution
Multi-SNP haplotype systems[a]					**M/M**		**M/m**		**m/m**			**MAF (%)**
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		*P*-values	OR
		SCZ	CON		
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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