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AN ASSOCIATION BETWEEN SEROTONIN RECEPTOR 3B GENE (*HTR3B*) AND TREATMENT-RESISTANT SCHIZOPHRENIA (TRS) IN A JAPANESE POPULATION

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ABSTRACT

Genetic factors are thought to be involved in the development of treatment-resistant schizophrenia (TRS). Since several antipsychotic drugs inhibit the release of neurotransmitters via the serotonin receptors 3 (5-HT3), a dysfunction of this kind of receptor might be associated with the development of TRS. Thus, single-marker and haplotype analyses of the tag-single nucleotide polymorphisms (SNPs) of the 5-HT3B subunit gene (*HTR3B*) were performed in TRS (n = 101) and non-TRS (n = 244) patients. The deletion allele at the 3 bp-insertion/deletion polymorphism site (–100_–102delAAG) located in the putative *HTR3B* promoter region is significantly more frequent in the TRS group than the insertion allele by a single-marker comparison (p=0.031). In addition, luciferase promoter assays showed that the deletion allele exhibited significantly higher transcriptional activity than the insertion allele in COS7 cells (p<0.05). These results suggest that *HTR3B* is involved in the development of TRS in the Japanese population.

Key Words: 5-HT3B subunit, Polymorphism, Treatment-resistant, Luciferase assay, Schizophrenia

INTRODUCTION

The prevalence of treatment-resistant schizophrenia (TRS) has been estimated at ~20–40%.[1)] Genetic factors in the inter-individual diversities in drug responsiveness have been shown to be involved in TRS,[2)] and several molecular genetic studies have been conducted to elucidate the genes underlying the predisposition to develop TRS.

Clozapine, an atypical antipsychotic drug, is reportedly effective in 30–60%[3)] of schizophrenic patients who are refractory to typical and other atypical antipsychotics.[4,5)] Among the several serotonin (5-HT) receptor subtypes, the 5-HT3 receptor is thought to play an important role in the responses of schizophrenic patients to clozapine and other antipsychotic drugs, since the drugs have been shown to reduce dopamine release by antagonizing the 5-HT3 receptor.[6)]

The 5-HT3B subunit of the 5-HT3 receptor is abundant in the caudate nucleus, hippocampus, thalamus, and amygdala, but is unable to form a functional homomeric receptor. However, when

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co-expressed with the 5-HT3A subunit, the two kinds of subunit form a heteromeric channel complex, that exhibits higher single-channel conductance than the homomeric 5-HT3A receptor.[7,8] The enhanced conductance is due to an HA-stretch region in the 5-HT3B subunit, whereas the 5-HT3B subunit may be unable to bind ligands as it lacks a principal binding site.[8,9] However, it reportedly modulates the binding at the 5-HT3A/5-HT3B receptor interface by providing a complementary binding site.[10] Taken together, the coexpression of the 5-HT3B subunit has striking effects on 5-HT3 receptor agonist and antagonist pharmacology.[11]

Because antipsychotic drugs can inhibit the release of several neurotransmitters via the 5-HT3 receptor in the brain,[12,13] the altered function of the 5-HT3B subunit might affect the heteromeric 5-HT3 receptor response to antipsychotics. Furthermore, the 5-HT3B subunit gene (*HTR3B*) is located on 11q23, which is one of the plausible candidate chromosomal regions for schizophrenia.[14]

Therefore, we considered *HTR3B* to be a candidate gene for the development of TRS. In this study, we investigated a possible association between the *HTR3B* polymorphisms and TRS using a single-marker association and haplotype analyses after evaluating the linkage disequilibrium (LD). In addition, the luciferase promoter assay of the polymorphism –100_–102delAAG associated with TRS was carried out in order to clarify the biological implications of this statistical significance.

MATERIALS AND METHODS

Subjects

This study was approved by the Ethics Committee of the Nagoya University School of Medicine, and written informed consent was obtained from each subject.

A total of 345 subjects (205 males and 140 females; age, mean and standard deviation, 54.1 ± 11.3 years; duration of illness, 33.6 ± 12.4 years; chlorpromazine-equivalent daily dose, 1021.1 ± 1857.1 mg/day) were selected from among schizophrenia patients who (1) met the Diagnostic and Statistical Manual of Mental Disorders (DSM)-IIIR criteria for schizophrenia, and (2) had received antipsychotic therapy for at least 1 year. Those defined as TRS patients were the 101 patients (67 males and 34 females; age, mean and standard deviation, 50.1 ± 10.5 years; age of onset of schizophrenia, 20.0 ± 5.3 years) who were receiving antipsychotic therapy at dosages of at least 1,000 mg chlorpromazine equivalent doses per day, while the remaining 244 were defined as non-TRS patients (138 males and 106 females; age, mean and standard deviation, 56.2 ± 11.9 years; age of onset of schizophrenia, 23.5 ± 8.2 years). These definitions were described in a previous report.[15]

Selection of haplotype-tagging SNPs

We first consulted the HapMap database (http://www.hapmap.org) and determined the LD blocks that fulfilled the criteria of $D' > 0.80$ and $r^2 > 0.80$ using HAPLOVIEW ver.3.2 software.[16] All the SNPs listed in the entire coding region, the 500-bp upstream 5′ flanking and the 500-bp downstream 3′ UTR regions, were included in the LD analysis. Using the same program, haplotype-tagged SNPs (htSNPs) were defined as those that captured 90% of the haplotype diversity within each LD block with no exclusion criterion of a minimum minor allele frequency. The Japanese portion of the HapMap data was used for this procedure. For *HTR3B*, gene constructed of three LD blocks, together with rs10502180, rs1176744 and rs3782025, were selected as the htSNPs used in this study with the criteria of $D' > 0.80$ and $r^2 > 0.80$ (Fig. 1). In addition to these 3 SNPs, we analyzed the –100_–102delAAG polymorphism within the

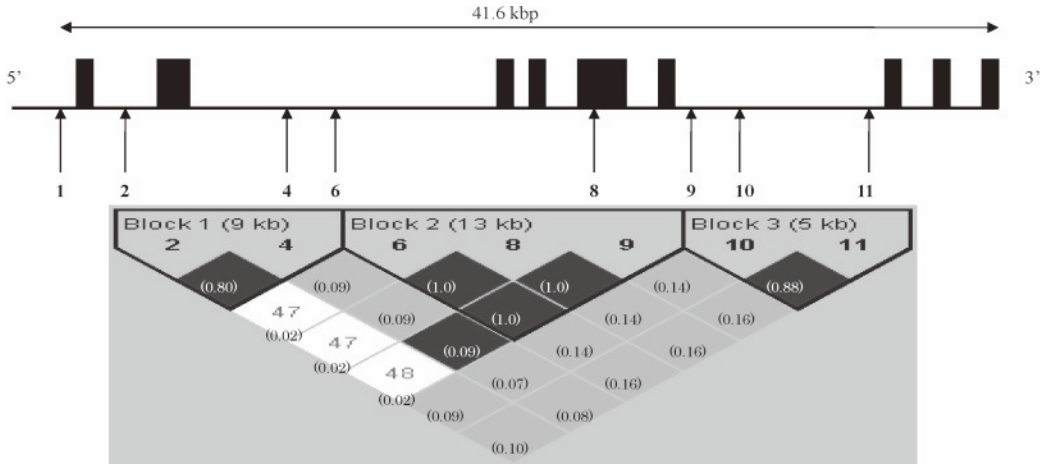

Fig. 1 Genomic structure of *HTR3B* and SNPs used in association analysis and LD mapping.
Vertical bars represent exons of *HTR3B*.
SNP ID: **1** –100_–102delAAG; **2** rs10502180; **4** rs12421126; **6** rs7103572; **8** rs1176744; **9** rs2276307; **10** rs3782025; **11** rs1672717.
※Numbers in boxes and parentheses represent D″ values and r^2 values after the decimal point, respectively. D′ values of 1.0 are not shown.

promoter region described by Tremblay *et al*,[17] which was previously reported to be associated with bipolar disorder.[18]

Genotyping

HtSNP genotyping was carried out by a polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP). The –100_–102delAAG polymorphism was determined using Gene Mapper software. Additional information regarding the primer sequences and the enzymes used in this study is available upon request.

Luciferase promoter assay

Three hundred-bp upstream fragments (-295 to 5 bp) of the initiation codon of the *HTR3B* that contained the –100_–102delAAG polymorphism were amplified by PCR and were digested by *Hin*dIII and *Nhe*I before cloning into the *Hin*dIII/*Nhe*I site of the pGL3-basic vector (Promega). The competent cell kit DH5a (TOYOBO, Tokyo Japan) was used to amplify the PGL3-*HTR3B* vectors containing the variant sequence. All the variant reporter constructs were sequenced to verify a successful mutagenesis. The amount of plasmid DNA was quantified using a NanoDrop ND-1000 UV-vis spectrophotometer.

The COS7 cells were cultured in Dulbecco's modified Eagle's medium with 10% fetal bovine serum and antibiotics at 37°C with 5% CO_2. For the assay, the cells were plated in 24-well plates at a density of 0.8×10^5 cells/well and cultured in an antibiotic-free medium for 24 hours before transfection. The transfection was carried out on the following day using Lipofectamine2000 reagent (Invitrogen). Briefly, one picomole of the luciferase reporter construct and 0.8 μg plasmid DNA were complexed with 2.0 μl of Lipofectamine2000 reagent in 500 μl Optimem I-reduced serum-free medium. After 5 hours of incubation, 1 ml of the complete medium was added. The transfected cells were further incubated at 37°C for 24 hours and lysed in 100 μl of passive

lysis buffer. The luciferase activity was measured using the PicaGene Luciferase Reporter Assay System (Toyo Ink., Tokyo, Japan) according to the manufacturer's instructions using MiniLumat LB9506 (Berthold, Germany).

Statistical analysis

Genotype deviation from Hardy-Weinberg equilibrium (HWE) was evaluated using the chi-square test. Both single-marker association analysis and haplotype analysis were performed with SPSS version 11.0 (Tokyo, Japan) and COCAPHASE version 2.403 (http://portal.litbio.org/Registered/Option/unphased), respectively. The observed genotype thus obtained regarding deviation from the HWE was evaluated using the method for an assessment of the deviation described by Wittke-Thompson *et al*.[19] This method is based on the chi-square goodness-of-fit approach, in which the distribution of any observed deviation from the HWE is tested in order to determine if the data fit the underlying genetic model. Since the Armitage trend test does not rely on an assumption of the HWE, it was used to statistically analyze the observed data that was unlikely to determine the best-fit model. The SNPSpD software program (http://genepi.qimr.edu.au/general/daleN/SNPSpD/) was used to correct for multiple testing. The luciferase promoter assay results were expressed as mean ± S.E. and were compared by one-way ANOVA and Student-Neuman-Keuls analysis. The level of significance for all statistical evaluations was set at 0.05.

RESULTS

The genotype and allele frequencies of the 4 polymorphisms (–100_–102delAAG, rs10502180, rs1176744, and rs3782025) in TRS and non-TRS subjects are summarized in Table 1. The observed genotype frequencies of rs1176744 and rs3782025 were within the distribution as expected according to the HWE. In a single-marker association analysis, we observed that the genotype frequency of the –100_–102delAAG polymorphism in the TRS group was significantly different from that in the non-TRS group (p = 0.031, corrected). Additionally, the minor allele frequency of the –100_–102delAAG(*del*) was significantly higher in the TRS than in the non-TRS

Table 1 Genotype and allelic distribution of *HTR3B* SNPs in TRS and non-TRS patients

SNP ID (M/N)	Genotype[a]						p-value[b]	Allele				p-value[b]
	TRS			non-TRS				TRS		non-TRS		
	M/M	M/m	m/m	M/M	M/m	m/m		M	m	M	m	
–100–102delAAG (*wt*/*del*)	70 (70%)	23 (23%)	8 (7%)	178 (74%)	59 (25%)	2 (1%)	**0.031**	163 (81%)	39 (19%)	415 (87%)	63 (13%)	**0.046**
rs10502180 (T/G)	72 (72%)	23 (23%)	6 (6%)	174 (71%)	56 (23%)	14 (6%)	0.974[c]	167 (83%)	35 (17%)	404 (83%)	84 (17%)	0.971[c]
rs1176744 (C/A)	63 (63%)	29 (29%)	9 (9%)	128 (53%)	89 (37%)	26 (10%)	0.255	155 (77%)	47 (23%)	345 (71%)	141 (29%)	0.133
rs3782025 (T/C)	49 (49%)	44 (44%)	7 (7%)	132 (55%)	92 (38%)	17 (7%)	0.593	142 (71%)	58 (29%)	356 (74%)	126 (26%)	0.45

a) M: major allele; m: minor allele; TRS: treatment-resistant schizophrenia

b) p-value was corrected by SNPSpD

c) p-value was tested by the Armitage trend test

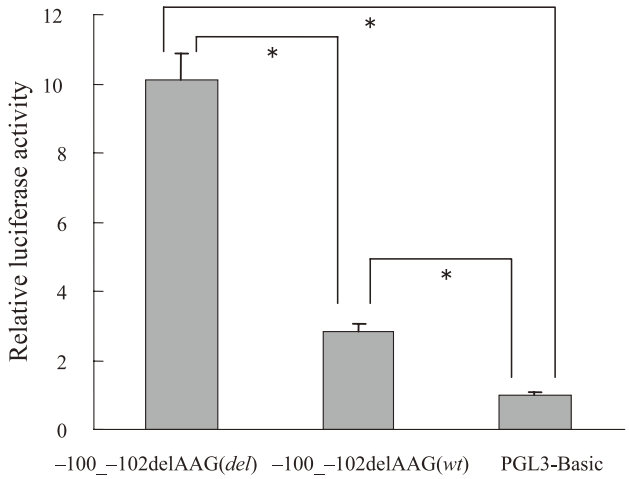

Fig. 2 Transcription activity of –100_–102delAAG polymorphism.
Results are expressed relative to PGL3-Basic and are the mean ± S.E. of data from 9 independent experiments. The 3 kinds of reporter plasmids carrying the –100_–102delAAG(*del*) promoter, –100_–102delAAG(*wt*) promoter and basic-vector displayed significantly different transcriptional activity among each other.
Asterisk (*) indicates a significant difference of $p < 0.05$ (Student-Neuman-Keuls analysis and One-way ANOVA)
Box-and-whisker bar indicates mean ± S.E.

group ($p = 0.046$). Regarding the luciferase promoter assay result, the reporter plasmid carrying the –100_–102delAAG(*wt*) promoter displayed lower transcriptional activity compared with the plasmid carrying the –100_–102delAAG(*del*) promoter, and approximately 3 times higher activity in comparison to the basic-vector plasmid (One-way ANOVA, $F(2, 24) = 95.126$, $p < 0.05$; Student-Newman-Keuls analysis, $p < 0.05$) (Fig. 2). These differences in transcriptional activity were consistent over 9 independent experiments.

DISCUSSION

We observed that the frequency of the *HTR3B* polymorphism (–100_–102delAAG) was significantly different between TRS and non-TRS patients. A 5-bp motif (*cis*-acting element) including the AAG deletion (AGAAG) was reported to bind the *trans*-acting heat shock factor (HSF), which could affect gene expression.[20] Therefore, we carried out the luciferase promoter assay of the polymorphism in order to clarify the biological implications of this statistical significance. As a result, without this sequence (–100_–102delAAG*del*), the *HTR3B* exhibited a higher level of expression.

The potency of the agonists to 5-HT3 was increased by coexpression of the 5-HT3B subunit.[11] Moreover, it has also been reported that doses of the receptor-antagonists required to elicit a response were significantly lower for the homomeric 5-HT3A receptor than for the heteromeric 5-HT3A/5-HT3B receptor.[7] Thus, the high level of *HTR3B* expression can increase the ratio of the heteromeric receptor, which weakens the homomeric 5-HT3A receptor response to the antagonists. The weakened effects of antipsychotic drugs through antagonism of the 5-HT3 receptor might lead to the development of TRS. In our luciferase assay result, the reporter plasmid

carrying the –100_–102delAAG(*del*) polymorphism exhibited higher activity. Additionally, those patients with the –100_–102delAAG(*del*) polymorphism are more likely to develop TRS. Thus, these data suggest that *HTR3B* may be involved in the development of TRS in the Japanese population.

Although a deviation from the HWE was observed, the –100_–102delAAG polymorphism was not excluded from the statistical analysis, since the deviation was tested in order to incorporate it into the underlying genetic model. The analysis showed a chi-square value of 1.43, which corresponds to $p > 0.05$, implying that the deviation from the HWE might be caused not by a genotype error but by the deletion sequence in the promoter region. This may easily give rise to an apparent deviation from the HWE[21] or the biological characteristics of this disease. Thus, the best-fit model is a good fit to the observed data.

In this study, the –100_–102delAAG(*del*) promoter region of the *HTR3B* has been provided to increase the 5-HT3B subunit expression in COS-7 cells. However, the functional explanation of this polymorphism in human specimens is still undetermined. Further transcription experiments will be needed in the future.

The criteria for TRS used in the present study were defined on the basis of patients' refractoriness to high doses of antipsychotic drugs that display a high affinity for dopaminergic systems. Although it is important to identify the genetic features of classical TRS subjects, the definition of TRS has been revised since the introduction of atypical antipsychotic drugs. TRS patients are diagnosed with TRS if they are unresponsive to 2 or more of the antipsychotic agents that show a high affinity for serotonergic systems. Further studies on such patients will be required.

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