Table 2. Genotypes and other clinical backgrounds associated with improvement rate in PANSS total score by a stepwise backward selection procedure.

Variable	p-value
Baseline PANSS total score (one-point increments)	0.0058
DRD2 -241A>G	0.0311
DRD2 TaqIA	0.0075
AKT1-SNP1 rs3803300	0.0183
AKT1-SNP5 rs2494732	0.0201

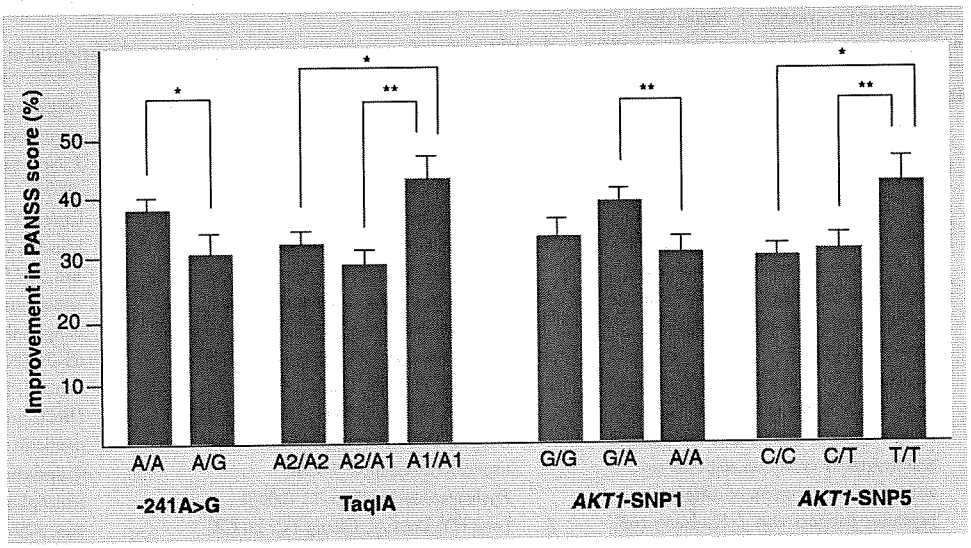
r^2: 0.25.
PANSS: Positive and Negative Syndrome Scale.

as a primary method because of weak LD between these variants (D′: 0.38; r^2: 0.045), but here we note that we undertook such an analysis but we could not confirm our previous findings (data not shown). The reasons for the discrepancies may derive from the background of the target samples; in our previous study, the subjects in the 'New' and 'Switched' groups were combined in order to increase the sample size, which might have caused a heterogeneous sample. By contrast, the present study included only first-episode, neuroleptic-naive subjects, resulting in pure phenotypes. In addition, the larger sample size in this study provided more statistical power than that in our previous study. In the previous report, TaqIA did not reach significance as a predictor (p = 0.08 [Yamanouchi & Iwata, Unpublished Data]), which might have been due to a type II error. However, a very likely explanation is that our previous positive report represented a type I error.

Concerning *AKT1*, it is an interesting finding that the identical variant, SNP5, may be a schizophrenia-susceptibility SNP [22] and a predictor SNP for risperidone treatment in schizophrenic patients. A recent pharmacogenetic study [25] showed no relationship between any of the tested SNPs in *AKT1* and therapeutic response to first- or second-generation antipsychotics. However, differences in LD among populations may also be responsible for such inconsistent results.

Emamian and colleagues showed that certain haplotypes (combination of SNP2 and 3) influenced the expression level of *AKT1* [19], but whether SNP1 or SNP5 are associated with function is unclear. To examine this, we developed a real-time reverse transcriptase PCR assay using lymphocyte-derived cell lines (25 subjects with schizophrenia and 25 age-matched healthy controls) to measure the expression of *AKT1* and *DRD2*. However, we found no evidence for association between any genotype and expression phenotype (data not shown). Since *AKT1* is an important intermediary between the D2 receptor and GSK3 proteins [19,20], *AKT1*-SNP5 and *AKT1*-SNP1 and/or SNPs in *DRD2* (-241A>G and TaqIA) may influence several signaling cascades in a gene–gene interaction manner.

Figure 1. Adjusted mean improvement rate in total PANSS score stratified by genotype in possible predictor SNPs.



Data shown are the mean ± SE. *p < 0.05; **p < 0.01.
PANSS: Positive and Negative Syndrome Scale.

Our study has a number of limitations. First, since we did not correct the p-values for multiple testing and sample size is relatively small, the results must be considered hypothesis generating and preliminary, and our nominally significant data need to be confirmed by independent samples. Second, we selected the patients who had completed 8 weeks of treatment, which might yield a false positive due to sampling bias as an open-label study. Third, we did not perform a comprehensive evaluation of any of the genes, for example, through a systematic LD-based study (e.g., tagging SNPs). Last, we did not detect the functional effect of these significant polymorphisms or did not include the fine mutation scan to search the actual causal variants. Therefore, the relationship between *DRD2* and *AKT1* polymorphisms and their functional relevance needs further study.

Conclusion & future perspective

We found predictor SNPs in *DRD2* and *AKT1* for risperidone treatment in schizophrenia patients. Further studies will be required for conslusive results, however, this evidence may lead to an improvement of response prediction and/or treatment selection for antipsychotics.

Acknowledgment

We thank M Miyata, S Ishihara, T Nakanishi and K Matsui for their technical support.

Financial & competing interests disclosure

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, the Core Research for Evolutional Science and Technology (CREST), and the Health Sciences Foundation (Research on Health Sciences focusing on Drug Innovation). The authors have no other relevant affiliations or financial involvement with any organization or entity with a financial interest in or financial conflict with the subject matter or materials discussed in the manuscript apart from those disclosed.

No writing assistance was utilized in the production of this manuscript.

Ethical conduct of research

The authors state that they have obtained appropriate institutional review board approval or have followed the principles outlined in the Declaration of Helsinki for all human or animal experimental investigations. In addition, for investigations involving human subjects, informed consent has been obtained from the participants involved

Executive summary

Risperidone response & dopamine- & serotonin-related genes

- In total, 120 first-episode neuroleptic-naive schizophrenic patients were examined to see if variants in dopamine-related genes (*DRD1–DRD5*, *AKT1* and *GSK3β*) and serotonin receptor genes (*HTR1A*, *HTR1B*, *HTR1D*, *HTR2A*, *HTR2C*, *HTR6* and *HTR7*) predicted the efficacy of risperidone treatment for schizophrenia.
- To reflect the gene–gene and gene–environmental interactions, multiple linear regression analysis was applied.

SNPs in DRD2 & AKT1 *were associated with risperidone response*

- Two SNPs in *DRD2* (-241A>G and TaqIA) and two SNPs in *AKT1* (*AKT1*-SNP1 and *AKT1*-SNP5) were significant predictors of treatment response to risperidone.
- These data suggest that SNPs in *DRD2* and *AKT1* may influence treatment response to risperidone in schizophrenia patients.

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Eur J Clin Pharmacol
DOI 10.1007/s00228-009-0633-8

PHARMACOGENETICS

Genetic and pharmacokinetic factors affecting the initial pharmacotherapeutic effect of paroxetine in Japanese patients with panic disorder

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Received: 22 November 2008 / Accepted: 6 February 2009

Abstract
Objective The objective of this study was to evaluate genetic and pharmacokinetic factors affecting the initial pharmacotherapeutic effect of paroxetine (PAX) in Japanese patients with panic disorder (PD).
Method Plasma concentration of PAX was determined by high performance liquid chromatography. Serotonin transporter gene-linked polymorphic region (5-HTTLPR) variants were determined by polymerase chain reaction techniques. PD severity was assessed using the Panic and Agoraphobia Scale (PAS).
Results Multiple regression analysis revealed that the plasma concentration of PAX, 5-HTTLPR genotype, and comorbid physical illness were significant factors affecting the initial pharmacotherapeutic effect of PAX in PD and indicated that these factors accounted for 52.4% (R^2=0.524) of the variability in the percent reduction in PAS score. The final model was described by the following equation (P=0.001): percent reduction in PAS score (%) = 68.5 − 1.2 × [plasma concentration of PAX (ng/ml)] − 33.0 × (L/S=1, S/S=0) − 21.8 × (with comorbid physical illness=1, without comorbid physical illness=0).
Conclusion The high plasma concentration of PAX, the L/S genotype of 5-HTTLPR, and comorbid physical illness might be associated with a poor response to the initial phase of pharmacotherapy of PD with PAX.

Keywords Pharmacotherapy · Panic disorder · Paroxetine · 5-HTTLPR · SSRIs

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Published online: 04 March 2009

Introduction

Panic disorder (PD) is characterized by sudden onset and repeated episodes of intense apprehension, fearfulness, or terror accompanied by physical symptoms that may include chest pain or discomfort, heart palpitations, shortness of breath, choking or smothering sensations, dizziness, or abdominal distress. The prevalence of PD has been estimated at 1–3% [1]. A gender difference has also been observed, with greater prevalence in females than in males [2]. Moreover, family and twin studies have suggested genetic liability for PD [3–5]. In a meta-analysis study, PD heritability was estimated to be about 48% [6].

Selective serotonin reuptake inhibitors (SSRIs) are thought to interact with the serotonergic nervous system and are believed to be effective for the treatment of PD. The efficacy of SSRIs including paroxetine (PAX) in the treatment of PD has been established in several placebo-

controlled trials [7–11], indicating that SSRIs have been recognized as the first-line agents for the treatment for PD.

Sandmann et al. investigated the relationship between the plasma level of fluvoxamine, one of the representative SSRIs, and the clinical response to fluvoxamine (50–300 mg/day, 1–5 weeks) in 16 PD patients in accordance with the DSM-III-R criteria [12]. In their study, six out of eight patients treated with fluvoxamine who showed full remission of panic attacks had a plasma level below 100 ng/ml [12], suggesting that increasing the plasma level of SSRIs might be associated with poor clinical effect in PD patients.

In accordance with the study of Sandmann et al. [12], Watanabe et al. reported that increasing the plasma concentration of PAX might be associated with poor clinical effect in PD patients [13]. Specifically, they investigated the clinical improvement of 21 unrelated Japanese patients who fulfilled the DSM-IV-TR criteria for a diagnosis of PD and who were treated with PAX (10 mg/day) for 2 weeks as initial treatment. Improvement of PD symptoms was assessed using the Panic and Agoraphobia Scale (PAS). In the range of plasma concentrations of PAX > 20 ng/ml, none of the subjects showed a percent reduction in PAS score > 20%. The subjects whose plasma concentration of PAX was < 20 ng/ml had a significantly higher mean percent reduction in PAS score than those whose plasma concentration of PAX was > 20 ng/ml. Multiple regression analysis showed that the plasma concentration of PAX was the only significant factor affecting the percent reduction in PAS score and accounted for 28.0% of the variability in the percent reduction in PAS score of the subjects. The final model of correlation was as follows: percent reduction of PAS score= $42.3-0.9\times$ [plasma concentration of PAX (ng/ml)] [R=0.529, P=0.014, coefficient of determination (R^2) = 0.280]. Assuming that the percent reduction in PAS score was 20% in the equation above, the plasma concentration of PAX is calculated to be about 25 ng/ml, which is suggested to be the upper end of the therapeutic window for the initial phase of PD treatment with PAX.

Meyer et al. reported a relationship between the serum concentration of PAX and the proportion of serotonin (5-HT) transporter (5-HTT) sites blocked. Striatal 5-HTT binding potential was measured with [^{11}C](N,N-dimethyl-2-(2-amino-4-cyanophenylthio) benzylamine ([^{11}C]DASB) and by positron emission tomography (PET) before and after 4 weeks of treatment with PAX. 5-HTT occupancy increased in a nonlinear fashion with the serum concentration of PAX such that its plateau of occupancy occurred at around 85% for a serum concentration of PAX > 28 ng/ml [14], suggesting no accumulation of clinical effect after reaching 28 ng/ml.

5-HTT removes serotonin from the synaptic cleft, and this protein is the primary target of action of SSRIs. The 5-HTT gene-linked polymorphic region (5-HTTLPR), which is located in the promoter region, has been identified as a functional polymorphism. The polymorphism consists of a 44-base-pair insertion or deletion involving repeat elements 6 to 8 [15]. In vitro, the basal activity of the long variant (L) was found to be more than twice that of the short variant (S) in 5-HTT mRNA synthesis and 5-HTT expression [15, 16]. These two different transcriptional efficiencies suggest that 5-HTT gene transcription is modulated by 5HTTLPR genetic variants [15, 16]. However, association studies have reported the absence of a significant difference in the 5-HTTLPR allele frequencies between PD subjects and normal controls [17–20].

Recent investigations have also been focused on the impact of genetic polymorphisms of 5-HTT on the clinical effect of SSRIs in PD because 5-HTT is the primary target of action of SSRIs. Perna et al. investigated the relationship between the allelic variation of 5-HTT and the clinical response to PAX in 92 PD patients who completed treatment with variable doses of PAX for 12 weeks. Both homozygotes for the long variant (L/L) of the 5-HTT promoter and heterozygotes (L/S) showed a better response to PAX than homozygotes for the short variant (S/S) (P< 0.03). This result was observed in the whole sample but was related to only female patients (P<0.02) [21].

In the present study, we investigated the association between therapeutic response to PAX 2 weeks after treatment initiation and the plasma concentration of PAX, 5-HTTLPR, and other clinical factors in Japanese PD patients.

Patients and methods

Patients

Thirty-eight unrelated Japanese patients who met the DSM-IV-TR criteria for a diagnosis of PD and who were receiving PAX (10 mg/day) participated in the present study. They were all drug-naïve outpatients at Dokkyo Medical University Hospital. The age of the patients (male=11, female=27) ranged from 21 to 72 years (mean±SD=34.3±9.8 years). Body weight ranged from 40 to 95 kg (57.3±11.2 kg). Six patients had comorbid major depressive disorder. The exclusion criteria of the present study were as follows: (1) axis I diagnosis other than PD and major depressive disorder, (2) presence of axis II diagnosis, (3) major laboratory abnormalities, (4) suicidal risk, (5) history of substance abuse, (6) use of antidepressants, antipsychotics, and benzodiazepines before the study, or (7) pregnancy. Written informed consent was obtained from each patient after the procedure was fully explained. The study protocol was approved by the Ethics Committee of Dokkyo Medical University Hospital.

Clinical evaluation, drug treatment regimen, and blood sampling

We performed routine laboratory tests including blood cell counts, liver and renal function tests, urinalysis, electrolyte and blood sugar measurements, and thyroid function tests during the first visit. The subjects were initially administered PAX (10 mg/day) at bedtime for 2 weeks as treatment for PD. Subjects with insomnia were prescribed brotizolam (0.25 or 0.5 mg) at bedtime (n=9), and those who had frequent panic attacks (n=15) were permitted to take a low dose of lorazepam (≤ 2.0 mg/day). PD severity was assessed using the PAS observer-rated version [22] at baseline and 2 weeks after the initiation of drug treatment. PAS has the advantage of being able to assess different aspects of PD separately using the five subscores of the scale (A: panic attacks, B: agoraphobic avoidance, C: anticipatory anxiety, D: disability, E: worries about health).

Patients were maintained on PAX for 2 weeks, and 7 ml of venous blood was collected 10–15 h after the last evening dose into Venoject tubes with EDTA-Na (Terumo Japan, Tokyo, Japan). Blood samples were centrifuged at 3,000 g for 10 min, and aliquots of the plasma and cell fraction were separated, frozen, and stored at −80°C until analysis.

Selection of subjects

Out of the 38 enrolled subjects, 8 showed plasma concentrations under the limit of detection, indicating poor compliance. These eight subjects were excluded from the analysis because the accurate value of the plasma concentration of PAX could not be determined since the inter- and intra-assay coefficients of variation (CVs) would be more than 20%, and accuracy is not assured below the limit of detection. There is a possibility that a very low concentration of PAX is observed in the subjects with gene duplication of *CYP2D6* [23, 24] because PAX is one of the representative substrates of CYP2D6; however, this is unlikely because the frequency of gene duplication of *CYP2D6* is very low in Japanese [25].

As shown in Table 1, a total of five subjects had adverse effects; two dropped out because of these effects, namely, daytime drowsiness (female, 25 years old, S/S genotype) and abnormal sensation (male, 39 years old, L/S genotype). Additionally, one subject refused blood collection just before sampling. Thus, a total of 11 subjects (8 subjects showed PAX concentration under the low limit of detection; 1 subject refused blood sampling; 2 subjects dropped out due to adverse effects) were excluded from the analysis. Accordingly, data from the remaining 27 subjects (male=6, female=21) were analyzed in the present study; their demographic data are shown in Table 2.

Table 1 Characteristics of subjects with adverse effects

Gender	Age	Adverse effect	5-HTTLPR genotype
Female	25	Daytime drowsiness	S/S
Female	31	Daytime drowsiness	L/S
Female	32	Daytime drowsiness	L/S
Female	46	Daytime drowsiness	S/S
Male	39	Abnormal sensation	L/S

Determination of plasma concentration of PAX

The plasma concentration of PAX was measured by column-switching high-performance liquid chromatography (HPLC) with ultraviolet detection, as developed by Hikida et al. [26]. Drugs in the plasma, to which cisapride had been added as an internal standard, were extracted with hexane-chloroform. The extract was subjected to an automated column-switching HPLC using a hydrophilic meta-acrylate polymer column for sample clean-up and a reversed-phase column for separation. The lowest limit of detection was 0.5 ng/ml, and the interassay CV was < 5% at 1 ng/ml PAX. The data on the plasma concentration of PAX have partly been analyzed and published in our previous report [13].

Genotyping

Genomic DNA was isolated from the blood-cell fraction using the QIAamp Blood kit (QIAGEN, Chatsworth, CA, USA). 5-HTTLPR genotypes (L and S alleles) were determined by polymerase chain reaction (PCR) techniques described by Lesch [15, 16] and Heilis et al. [16] with minor modification. Oligonucleotide primers flanking the 5-HTTLPR and corresponding to the nucleotide positions ranging from −1416 to −1397 (LPR5; 5'-GGCGTTGCC GCTCTGAATTGC) and from −910 to −889 (LPR3; 5'-GAGGGACTGAGCTGGACAACCCAC) of the 5-HTT gene regulatory region were used to generate a 484/528-bp fragment. PCR amplification was carried out in a final volume of 12.5 μl consisting of 20 ng of genomic DNA, 0.8 mM dNTP mixture, 0.05 μg of sense and antisense primers (i.e., LPR5 and LPR3), 1×PCR buffer, 1.5 mM $MgCl_2$, 5% dimethyl sulfoxide, and 0.5 U of AmpliTaq DNA polymerase (Applied Biosystems Japan, Tokyo, Japan). Annealing was performed at 60°C for 30 s, extension at 72°C for 1 min, and denaturation at 94°C for 30 s for 35 cycles.

Statistical analysis

For statistical analysis we used linear regression analysis, multiple regression analysis, and Fischer's exact probability test using SPSS version 12.0 (SPSS Japan, Tokyo, Japan)

Table 2 Demographic characteristics at baseline according to 5-HTTLPR genotypes

5-HTTLPR genotypes	L/S	S/S	Total
Number of patients	9	18	27
Male/female	1/8	5/13	6/21
Age (years)	34.4±7.6	34.9±12.7	34.7±11.1
Body weight (kg)	56.0±9.8	54.4±8.4	54.9±8.7
With major depressive disorder	3	2	5
With/without agoraphobia	6/3	15/3	21/6
Panic attacks/week	1.8±1.3	2.7±4.3	2.4±3.6
Panic and agoraphobia scale	21.7±6.7	23.0±6.1	22.6±6.2

Values are presented as number or mean ± SD

and Prism version 2.0 (GraphPad, San Diego, CA, USA). P values < 0.05 were considered significant.

Results

The mean PAS score was 21.6±6.9 (range, 9–34) at baseline before the initiation of pharmacotherapy with PAX. After 2 weeks of treatment, the mean PAS score improved to 15.1±6.9 (2–31). The mean percent reduction in PAS score (%) [percent reduction in PAS score = ([PAS score at baseline – PAS score 2 weeks after the initiation of PAX treatment)/PAS score at baseline) × 100) was 31.4± 27.9% (−9.1 to 86.2%).

Figure 1 shows the relationship between the percent reduction in PAS score and the plasma concentration of PAX 2 weeks after the initiation of PAX administration. There was an approximately 23-fold interindividual variation in the plasma concentration of PAX (2.6–59.3 ng/ml) and also a large interindividual variation in the percent reduction in PAS score (−9.1 to 86.2%). There was a significant negative correlation between the percent reduction in PAS score and the plasma concentration of PAX irrespective of genotypes (R=0.42, P= 0.02). Moreover, there was a significant negative correlation between the percent reduction in PAS score and the plasma concentration of PAX in subjects with the S/S genotype of 5-HTTLPR (R=0.61, P=0.006), while no significant correlation between the percent reduction in PAS score and the plasma concentration of PAX in those with the L/S genotype of 5-HTTLPR was observed (R= 0.33, P=0.37).

As stated in the "Introduction" section, a PAX concentration of 28 ng/ml is suggested to be the upper end of the optimal range of PAX concentration from a PET study [14]. As shown in Fig. 1, there were no subjects with PAX concentration > 28 ng/ml showing a percent reduction of PAS score > 20%. In the PAX concentration range under 28 ng/ml, the subjects (92.9%) with the S/S genotype were more likely to show a percent reduction in PAS score > 20% than those with the L/S genotype [see Fig. 1; 13 out of 14 subjects (92.9%) vs 4 out of 9 subjects (44.4%), Fisher's exact probability test, P=0.018].

Multiple regression analysis was performed in order to analyze the relationship between demographic variables from the subjects and the clinical response to PAX (percent reduction in PAS score). We used the plasma concentration of PAX, age, gender, body weight, comorbid physical illness, comorbid major depressive disorder, comorbid agoraphobia, smoking status, habitual use of alcohol, PAS score at baseline, frequency of panic attacks per week at baseline, 5-HTTLPR genotype (L/S or S/S), adverse effect of PAX, use of lorazepam and/or brotizolam as independent variables and the clinical response to PAX (percent reduction in PAS score) as the dependent variable. Plasma concentration of PAX, 5-HTTLPR genotype, and comorbid physical illness were

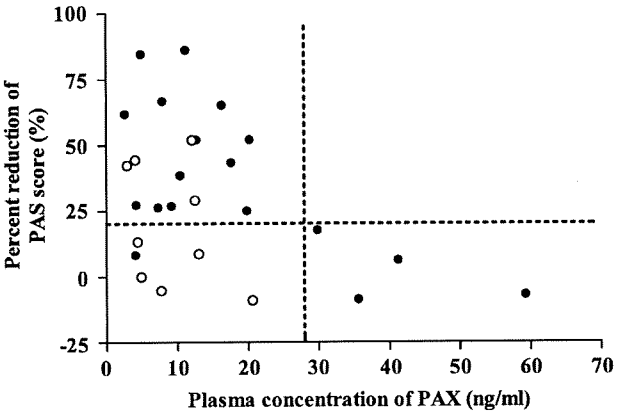

Fig. 1 Relationship between percent reduction in PAS score (*ordinate*), plasma concentration of PAX (*abscissa*) and 5-HTTLPR genotype (*empty circles* = subjects with the L/S genotype; *filled circles* = subjects with the S/S genotype) 2 weeks after the initiation of PAX administration. *Horizontal* and *vertical dashed lines* indicate 20% reduction in PAS score and PAX plasma concentration of 28 ng/ml, respectively. Note that a negative correlation between the percent reduction in PAS score and the plasma concentration of PAX was observed, and no subjects with a PAX concentration > 28 ng/ml showed a percent reduction in PAS score > 20%. In the PAX concentration range under 28 ng/ml, 13 out of 14 subjects (92.9%) with the S/S genotype showed a percent reduction in PAS score > 20%, while only 4 out of 9 subjects (44.4%) with the L/S genotype showed a percent reduction in PAS score > 20% (Fisher's exact test, P=0.018)

found to be significant factors affecting the percent reduction in PAS score (see Table 3); the correlation coefficient (R) for the full model was 0.724, indicating that these factors accounted for 52.4% (R^2=0.524) of the variability in the clinical response to PAX. The final model was thus described by the following equation (P=0.001): percent reduction in PAS score (%) = 68.5 − 1.2 × [plasma concentration of PAX (ng/ml)] − 33.0 × (L/S=1, S/S=0) − 21.8 × (with comorbid physical illness=1, without comorbid physical illness=0) (see Table 3).

Discussion

Stahl reported that PD patients tend to be more sensitive to SSRIs than depressed patients, since they can easily develop short-term worsening of their symptoms when pharmacotherapy is initiated [27]. Thus, PD patients are usually started at a lower dose than depressed patients [27]. Louie et al. reported that PD patients with accompanying major depressive disorder showed lower tolerability to SSRIs than patients with major depressive disorder alone [28]. Gilles et al. reported different upper thresholds of serum concentrations along the progression of pharmacotherapy in major depression, and they speculated that a high serum concentration of SSRIs may lead to 5-HTergic side effects such as anxiety or disturbances in sleep, appetite, and sexual function, which may impede the overall response to pharmacotherapy with SSRIs [29].

Table 3 Results of stepwise multiple regression analysis

Independent variable	P
Gender	0.386
Age	0.350
Body weight	0.428
Comorbid major depressive disorder	0.405
With/without agoraphobia	0.618
Smoking status	0.478
Habitual use of alcohol	0.929
PAS score at baseline	0.216
Initial panic attacks per week at baseline	0.965
Use of lorazepam and/or brotizolam	0.761
Adverse effect	0.603
Plasma concentration of paroxetine	0.001
5-HTTLPR genotype	0.001
Comorbid physical illness	0.016

The final model was described by the following equation (P=0.001): percent reduction in PAS score (%) = 68.5 − 1.2 × [plasma concentration of PAX (ng/ml)] − 33.0 × (L/S=1, S/S=0) − 21.8 × (with comorbid physical illness=1, without comorbid physical illness=0) [R=0.724, P=0.001, coefficient of determination (R^2) = 0.524]

In the present study, the plasma concentration of PAX has been shown to be one of the important determinants of the initial clinical response and a high plasma concentration of PAX has been shown to impede such a clinical response. Why does a high plasma concentration of PAX impede a favorable clinical response at the initial phase of pharmacotherapy (2 weeks) in PD? The cell bodies of 5-HT neurons are located in the brainstem area, that is, the raphe nucleus, and projections from the raphe nucleus to the frontal cortex are thought to be important for regulating mood. Projections from the raphe nucleus to the amygdala and prefrontal cortex may also play an important role in inhibiting anxiety. 5-HTergic terminals from the raphe nucleus to the prefrontal cortex inhibit emotional input from the prefrontal cortex to the amygdala [30]. 5-HTergic terminals from the raphe nucleus to the amygdala act as brakes on outputs from the amygdala to a fear response [30]. Thus, treatment with SSRIs has dual processes in that it diminishes the precipitation of both anxiety and fear [30]. SSRIs inhibit 5-HTT, leading to an increase in the amount of 5-HT in the synaptic cleft. The increase in 5-HT in the somatodendritic area of the 5-HT neurons causes desensitization or down-regulation of somatodendritic 5-HT_{1A} autoreceptors, and following the down-regulation of these autoreceptors, the 5-HT neuronal impulse flow is increased. This inhibits both the emotional input from the prefrontal cortex to the amygdala and the output from the amygdala to a fear response.

However, at the initial phase of pharmacotherapy with SSRIs, particularly before the down-regulation of 5-HT_{1A} autoreceptors, the amount of 5-HT increases to a higher level at the cell body area in the raphe nucleus than in the axon terminals [30]. 5-HT_{1A} receptors, which are autoreceptors located on cell bodies and dendrites, detect the increase in 5-HT and cause a shutdown of the 5-HT neuronal impulse flow before the desensitization, which might lower the activity of the 5-HT projection to both the prefrontal cortex and the amygdala from the raphe nucleus.

Accordingly, the increase in 5-HT in the somatodendritic area is indispensable for desensitizing the 5-HT_{1A} receptors and for inhibiting PD symptoms; however, the increase in 5-HT in the somatodendritic area before the down-regulation of 5-HT_{1A} receptors may lead to the deterioration of PD symptoms, which might be caused by a high plasma concentration of PAX.

Perna et al. reported that both the L/L genotype and the L/S genotype of 5-HTTLPR showed a significantly better response to PAX than the S/S genotype in female PD patients 12 weeks after the initiation of pharmacotherapy [21]. In contrast to the report of Perna et al. [21], the patients with the L/S genotype of 5-HTTLPR showed a lower clinical response than those with the S/S genotype in the present study. The difference in the results between the

two studies might be due to the difference in the observation duration, i.e., 2 weeks in the present study vs. 12 weeks in the study of Perna et al. [21].

Stahl reported that SSRIs cause a shutdown of the 5-HT neuronal impulse flow before the desensitization of 5-HT_{1A} autoreceptors, which might lead to the decline of PD symptoms [27]. Consequently, L allele carriers were assumed to be more sensitive to the pharmacological effects of PAX than those with the S/S homozygote.

In the present study, the therapeutic response of the subjects with the S/S genotype to PAX was better than that of the subjects with the L/S genotype 2 weeks after the initiation of pharmacotherapy with PAX. In order to explain the present finding on the 5-HTTLPR genotypes, we must speculate about the status of 5-HT_{1A} receptors, which are autoreceptors located on cell bodies and dendrites, in drug naïve patients.

David et al. used ^{11}C-*N*-(2-(4-(2-methoxyphenyl)-1-piperazinyl)ethyl)-*N*(2-pyridyl)cyclohexanecarboxamide ([^{11}C]-WAY) as a selective radioligand for the 5-HT_{1A} receptor and reported that the 5-HT_{1A} receptor genotype showed no significant effects on 5-HT_{1A} receptor binding in a PET study. On the other hand, 5-HT_{1A} receptor binding potential values were lower in all brain regions including the raphe nucleus in healthy subjects with the S/S or L/S genotype of 5-HTTLPR than in healthy individuals with the L/L genotype [31]. When the same process occurred in the drug-naïve patients with PD, the drug-naïve patients with the S/S genotype of 5-HTTLPR showed decreased 5-HTT function, and this condition might lead to a lifelong increase in the concentration of 5-HT in the synaptic cleft. Additionally, the 5-HT_{1A} receptor in the drug-naïve patients with the S/S genotype of 5-HTTLPR is thought to be, as it was proven, "down-regulated" compared with the drug-naïve patients with the L/S genotype.

In the present study, comorbid physical illness was shown to be associated with a poor response to PAX. The presence of medical comorbidity complicates the identification, presentation, and treatment of PD because a number of physical illness including cardiovascular disease, respiratory disorders, and vestibular and thyroid dysfunctions have symptoms that overlap with symptoms of PD [32].

As shown in Table 1, two subjects with the S/S genotype and three subjects with the L/S genotype reported adverse effects. Understanding the relationship between the 5-HTTLPR genotype and the development of adverse effects has been controversial. Murphy et al. reported that S allele carriers experienced more severe adverse events during pharmacotherapy with PAX [33]. Perlis et al. reported that the S allele may be used to identify patients at risk for developing insomnia or agitation with fluoxetine treatment [34]. In contrast, Takahashi et al. reported no association between 5-HTTLPR and the development of nausea during treatment with fluvoxamine [35]. Moreover, Kato et al. reported no association between 5-HTTLPR and adverse effects during treatment with fluvoxamine and PAX [36].

The present study has several limitations. Firstly, only one genetic polymorphism, i.e., 5-HTTLPR, was assessed. PD is considered to be a polygenic disorder, and it is believed that other genes such as 5-HT receptor genes and progesterone receptor genes might contribute to the pathogenesis of PD. Secondly, this study analyzed a relatively small sample size due to the high drop-out rate. This resulted in having no subjects with the L/L genotype, because the frequency of the L/L genotype in Japanese has been reported to be approximately only 3% [37]. Thirdly, the present analysis has been limited to only the early phase of the pharmacotherapy for PD, i.e., 2 weeks after the initiation of pharmacotherapy. Since PD is a chronic illness, it would also be important to determine the clinical response in the later phase of pharmacotherapy. Accumulation of data on the clinical response of PD to PAX in the later phase of pharmacotherapy is in progress.

In conclusion, high plasma concentration of PAX, 5-HTTLPR L/S genotype, and comorbid physical illness are associated with a poor response of PD to PAX in the initial phase of pharmacotherapy.

Acknowledgements This research was supported by KAKENHI, Grants-in-Aid for Scientific Research, and a Grant-in-Aid for Young Scientists from the Japan Society for the Promotion of Science (JSPS) (#18591307) and the Ministry of Education, Culture, Sports, Science and Technology (MEXT) (#19790837 and #18790847). The authors are grateful to Ms. Yoshimi Aoyama for her excellent technical assistance. The authors have no conflicts of interest directly relevant to the content of the present study.

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J Neural Transm (2008) 115:1347–1354
DOI 10.1007/s00702-008-0100-1

BIOLOGICAL PSYCHIATRY - ORIGINAL ARTICLE

Failure to confirm an association between *Epsin 4* and schizophrenia in a Japanese population

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Received: 28 March 2008 / Accepted: 20 July 2008 / Published online: 12 August 2008

Abstract Previous studies suggested that genetic variations in the 5′ region of *Epsin 4*, a gene encoding enthoprotin on chromosome 5q33, are associated with schizophrenia. However, conflicting results have also been reported. We examined the possible association in a Japanese sample of 354 patients and 365 controls. Seventeen polymorphisms of *Epsin 4* [3 microsatellites and 14 single nucleotide polymorphisms (SNPs)] were selected. A microsatellite marker (D5S1403) demonstrated a significant difference in the allele frequency between patients and controls (uncorrected $P = 0.04$). However, there was no significant difference in the genotype or allele frequency between the two groups for the other microsatellites or SNPs. Haplotype-based analysis provided no evidence for an association. The positive result at D5S1403 no longer reached statistical significance when multiple testing was taken into consideration. Our results suggest that the examined region of *Epsin 4* does not have a major influence on susceptibility to schizophrenia in Japanese.

Keywords Association study · *Epsin 4* · Enthoprotin · Schizophrenia · Polymorphism

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Introduction

Schizophrenia is a debilitating psychiatric disorder that affects approximately 1% of the world's population (Fenton et al. 2003; Hyman 2000). Patients may suffer from delusions, hallucinations, disorganized speech and behavior, as well as impairment in short-term verbal and nonverbal memory. The complete etiology of the disease remains unknown, though twin and adoption studies have demonstrated that schizophrenia is highly heritable (estimated heritability of >80%) (Cardno et al. 1999). While the contributing genes and pathophysiological mechanisms remain elusive, identifying the susceptibility genes is essential in discovering the true pathogenesis of schizophrenia. The mode of schizophrenia transmission is complex and is thought to be polygenic (Owen et al. 2004). Thus far, linkage and association studies have been successful approaches in searching for complex disease genes, discovering such candidate genes as neuregulin 1, dysbindin, G72, and D-amino acid oxidase (DAAO). Through such analysis, several chromosomal regions have been identified and investigated as potential sources for schizophrenia susceptibility genes (Chumakov et al. 2002; Stefansson et al. 2002; Straub et al. 2002a). As a result, the long arm of chromosome 5q has been identified as a putative chromosomal region of interest and subsequently investigated for susceptibility loci (Lewis et al. 2003; Straub et al. 2002b).

An association study of English, Irish, Welsh, and Scottish populations found *Epsin 4* on chromosome 5q33 as a strong candidate for schizophrenia susceptibility (Pimm et al. 2005). Four associated polymorphisms were discovered at the 5′ end of *Epsin 4*, a gene encoding the clathrin-associated protein enthoprotin. These included two microsatellite markers, D5S1403 and AAAT11, and

two single-nucleotide-polymorphism (SNP) markers, rs10046055 and rs254664. Enthoprotin plays a critical role in the formation, transport and stability of clathrin-coated vesicles (CCVs) and is therefore thought to regulate the transport and storage of neurotransmitters in the brain (McPherson and Ritter 2005; Wasiak et al. 2002). Neuronal CCVs are critical in the insertion and recycling of neurotransmitter receptors at the postsynaptic membrane and have been implicated as a regulatory mechanism for synaptic plasticity (Blanpied et al. 2002; Wang and Linden 2000). Specifically, CCVs facilitate AMPA receptor trafficking on the postsynaptic membrane, therefore affecting overall glutamatergic neurotransmission (Malinow and Malenka 2002; Man et al. 2002). Dysfunction of enthoprotin could, therefore, stimulate disturbances in glutamatergic brain signaling as well as synaptic plasticity, both postulated to be integral components of schizophrenia pathophysiology (Christison et al. 1989; Carter et al. 2006). Moreover, it has been demonstrated that enthoprotin interacts with the soluble *N*-ethylmaleimide-sensitive factor attachment protein receptor (SNARE) complex found in hippocampal pyramidal cells (Antonin et al. 2000; Chidambaram et al. 2004). This suggests a possible role for enthoprotin in calcium-mediated vesicle fusion of excitatory neurotransmitters released from the hippocampus. If the SNARE complex does not function properly in this way, abnormal neural connectivity may result, which is another characteristic of schizophrenia (Honer et al. 2002).

Thus far, two replication studies have been conducted in the Han Chinese population (Liou et al. 2006; Tang et al. 2006). Both studies found no association between the four markers previously reported (D5S1403, AAAT11, rs10046055, or rs254664) and schizophrenia. Tang et al. (2006), however, did detect haplotypes near the 5′ end of *Epsin 4* (252/T consisted of AAAT11 and rs10046055, global $P = 0.0021$; T/T of rs1145603 and rs254664, $P = 0.0033$) showing an association with schizophrenia in Han Chinese family trios. Liou et al. (2006) analyzed nine SNPs on *Epsin 4* in a case–control design and found a significant difference in the allele frequency of a SNP on the 5′ upstream region of *Epsin 4* (rs1186922); however, this difference was not significant after multiple testing. These conflicting results prompted us to examine *Epsin 4* for an association with schizophrenia in a Japanese sample.

Materials and methods

Subjects

Subjects were 354 patients with schizophrenia [212 males, mean age of 44.0 years (SD 13.7)] and 365 healthy controls [113 males, mean age of 39.7 years (SD 14.1)]. All subjects were biologically unrelated Japanese and recruited from the same geographical area (Western part of Tokyo Metropolitan). Consensus diagnosis by at least two psychiatrists was made for each patient according to the Diagnostic and Statistical Manual of Mental Disorders, 4th edition (DSM-IV) criteria (American Psychiatric Association 1994) on the basis of unstructured interviews and information from medical records. The controls were healthy volunteers recruited from hospital staffs and their associates. Control individuals were interviewed and those with a current or past history of psychiatric treatment or regular use of psychotropic agents were not enrolled in the study. Participants were excluded from both the patient and control groups if they had prior medical histories of central nervous system disease or severe head injury, or if they met criteria for alcohol/drug dependence or mental retardation. The study protocol was approved by the ethics committee at the National Center of Neurology and Psychiatry, Japan. After description of the study, written informed consent was obtained from every subject.

Genotyping

Venous blood was drawn from the subjects and Genomic DNA was extracted from peripheral leukocytes using the Wizard Genomic DNA Purification System kit (Promega, Madison, WI, USA) based on the solution-based method according to the manufacturer's instructions. Fourteen SNPs (rs1186922, rs10046055, rs1894962, rs6556290, rs7735412, rs1145585, rs1186930, rs1145603, rs1186934, rs11744778, rs1145602, rs1186998, rs17055032, rs254664) and three microsatellites (D5S1400, D5S1403, AAAT11) were genotyped. Five SNPs (rs1186922, rs10046055, rs1186930, rs1145603, rs254664) were selected based on previous studies conducted by Liou et al. (2006), Tang et al. (2006), and Pimm et al. (2005). The three microsatellites demonstrated to be significantly associated with schizophrenia by Pimm et al. (2005) and Tang et al. (2006) were selected in order to replicate their positive findings. The other nine SNPs (rs1894962, rs6556290, rs7735412, rs1145585, rs1186934, rs11744778, rs1145602, rs1186998, rs17055032) were selected from the international HapMap project (http://hapmap.org/index.html.en) using Paul de Bakker's Tagger algorithm in the Haploview V 3.32 program. The 17 studied polymorphisms cover approximately 193 kb of *Epsin 4* from the 5′ upstream region to intron 1 (Fig. 1).

The SNPs were genotyped using the TaqMan 5′-exonuclease allelic discrimination assay. TaqMan probes of the assay on demand (rs1186922, rs10046055, rs6556290, rs7735412, rs1145585, rs1186930, rs1145603, rs1186934, rs11744778, rs1145602, rs1186998, rs17055032, rs254664)

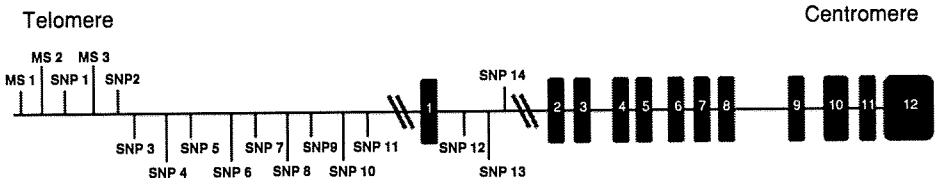

Fig. 1 The genetic structure of *Epsin 4* and location of studied markers. Upper six markers were studied in the previous reports. Lower 11 markers were additionally selected for Tag SNPs in this area

and assay by design (rs1894962) with Universal PCR Master Mix were obtained from Applied Biosystems (Foster City, CA, USA). Thermal cycling conditions for polymerase chain reaction (PCR) were one cycle at 95°C for 10 min followed by 50 cycles of 92°C for 15 s and 60°C for 1 min. After amplification, the allelic specific fluorescence was measured on ABI PRISM 7900 Sequence Detector Systems (Applied Biosystems, Foster City, CA, USA). Genotype data were read blind to the case–control status. Ambiguous genotype data were not included in the analysis.

Primer sequences for PCR amplification of the microsatellite markers D5S1400 and D5S1403 were obtained from the uniSTS database in NCBI (http://www.ncbi.nlm.nih.gov/), while the primer sequence of microsatellite marker AAAT11 was obtained from Pimm et al. (2005). PCR amplification of microsatellite markers was performed using primers fluorescently labeled with Beckman dyes. PCR products were subject to electrophoresis on Beckman CEQ 8000 (Beckman Coulter, Fullerton, CA, USA). As the PCR fragments of microsatellites were not the same length as those in the original work, we adjusted the sizes of our PCR fragments to Pimm et al. (2005).

Statistical analysis

Deviations of genotype distributions from Hardy–Weinberg equilibrium were assessed with the χ^2 test for goodness of fit. Genotype and allele distributions of each SNP were compared between patients and controls by using the χ^2 test for independence. These tests were performed with the SPSS software ver. 11 (SPSS Japan, Tokyo, Japan). The allelic association of microsatellite markers with schizophrenia was examined by use of the CLUMP program (Sham and Curtis 1995), which assesses the significance of departure between the observed and expected values in a $2 \times N$ contingency table using a Monte Carlo approach. The standardized measure of linkage disequilibrium (LD), (D') and r^2, were estimated using the online software SHEsis (http://202.120.7.14/analysis/myAnalysis.php). Haplotype-based association analyses were examined with the COCAPHASE software ver. 2.4 (http://www.mrc-bsu.cam.ac.uk/personal/frank/software/unphased/) (Dudbridge et al. 2000). The expectation–maximization (EM) and "droprare" options were used. Haplotypes with frequencies less than 3% were considered to be rare.

Results

All investigated SNPs and microsatellite markers for case and control groups resulted in distributions that did not significantly deviate from Hardy–Weinberg Equilibrium. LD estimates of pairwise markers, expressed in D' and r^2, are presented in Table 1. Pairs in LD are represented as gray-shaded values, with estimates of $D' > 0.8$ and $r^2 > 0.8$. Pairwise LD analysis and haplotype block determination demonstrated that there were two blocks across the studied genomic region (Fig. 2). Measurement of pairwise LD showed that the middle seven SNPs (rs1145585, rs1186930, rs1145603, rs1186934, rs11744778, rs1145602, and rs1186998) were in strong LD with each other and were located in the sample block (haplotype block 2). The remaining block, haplotype block 1, consisted of three SNPs (rs1186922, rs10046055, and rs1894962) in the 5′ upstream region. In the haplotype-based analysis, the haplotypes in the two blocks were analyzed separately.

In the single-marker analysis, the microsatellite 2 marker (D5S1403) demonstrated a significant difference in allele frequency between case and control groups (Table 2: Allele 203, $\chi^2 = 4.26$, $df = 1$, $P = 0.04$ from the "T3" analysis of CLUMP). The remaining microsatellite markers and SNPs did not show any significant allelic association with schizophrenia (Table 2, all $P > 0.05$). The obtained evidence for an association at microsatellite 2 with schizophrenia was weak and non-significant when multiple testing was taken into consideration.

Results of the haplotype-based analysis are also presented in Table 2. None of the haplotypes showed a statistically significant difference between case and control groups. Even when haplotypes were examined according to the groupings established by Pimm et al. (2005), no evidence for an association was obtained. Tang et al. (2006) reported a highly significant transmission of haplotypes consisting of AAAT11 and rs10046055 ($P = 0.0048$) in addition to rs254664 and rs1145603 ($P = 0.0047$) to affected offspring in Han Chinese trios. Our results, again, failed to provide evidence to support this finding.

Table 1 Pair-wise linkage disequilibrium in *Epsin 4*

		MS1 D5S1400	MS2 D5S1403	SNP1 rs1186922	MS3 AAAT11	SNP2 rs10046055	SNP3 rs1894962	SNP4 rs6556290	SNP5 rs7735412	SNP6 rs1145585	SNP7 rs1186930	SNP8 rs1145603	SNP9 rs1186934	SNP10 rs11744778	SNP11 rs1145602	SNP12 rs1186998	SNP13 rs17055032	SNP14 rs254664
MS1	D5S1400		0.071	0.123	0.399	0.347	0.216	*0.979*	0.207	0.026	0.030	0.031	0.038	0.337	0.046	0.087	0.336	0.344
MS2	D5S1403	0.001		0.309	0.250	0.235	0.310	*0.981*	0.246	0.102	0.240	0.243	0.237	0.293	0.114	0.304	0.090	0.294
SNP1	rs1186922	0.005	0.051		*0.879*	*0.999*	*1.000*	*0.999*	0.465	*0.861*	0.579	0.581	0.566	0.032	*0.813*	0.717	0.347	0.062
MS3	AAAT11	0.011	0.014	0.045		*0.897*	0.757	*1.000*	0.741	0.673	0.426	0.430	0.434	0.442	0.668	0.633	0.307	0.441
SNP2	rs10046055	0.008	0.013	0.063	0.775		*0.999*	*1.000*	0.589	*0.993*	0.487	0.490	0.508	0.502	0.581	0.707	0.400	0.516
SNP3	rs1894962	0.007	0.004	0.425	0.015	0.027		0.053	*0.989*	0.772	0.720	0.721	0.699	*0.859*	0.716	*0.855*	0.136	*0.927*
SNP4	rs6556290	0.002	0.004	0.008	0.001	0.001	0.000		*1.000*	0.257	*0.997*	*0.996*	*0.998*	*1.000*	0.259	*0.998*	*1.000*	1.000
SNP5	rs7735412	0.001	0.002	0.013	0.002	0.001	0.003	0.000		*0.923*	*1.000*	*0.999*	*1.000*	*0.955*	*0.835*	*0.999*	*0.987*	*1.000*
SNP6	rs1145585	0.000	0.002	0.415	0.015	0.035	0.455	0.001	0.004		*1.000*	*0.985*	*0.970*	*0.912*	*0.942*	*0.982*	*0.989*	*0.999*
SNP7	rs1186930	0.000	0.041	0.251	0.065	0.092	0.165	0.006	0.046	0.416		*1.000*	*0.997*	*0.984*	*0.931*	*0.992*	*0.996*	*1.000*
SNP8	rs1145603	0.000	0.043	0.254	0.067	0.094	0.166	0.006	0.046	0.406	*0.994*		*0.997*	*0.984*	*0.932*	*1.000*	*0.996*	*1.000*
SNP9	rs1186934	0.000	0.042	0.243	0.068	0.101	0.159	0.006	0.044	0.399	*0.980*	*0.987*		*0.984*	*0.939*	*0.996*	*0.995*	*0.992*
SNP10	rs11744778	0.009	0.035	0.000	0.185	0.249	0.020	0.000	0.110	0.029	0.372	0.375	0.375		*0.999*	*0.991*	0.509	*0.978*
SNP11	rs1145602	0.000	0.003	0.368	0.014	0.012	0.395	0.001	0.003	*0.883*	0.363	0.365	0.374	0.034		*0.937*	0.692	*0.919*
SNP12	rs1186998	0.003	0.050	0.295	0.111	0.150	0.179	0.005	0.036	0.309	0.759	0.766	0.755	0.292	0.284		*1.000*	*1.000*
SNP13	rs17055032	0.003	0.000	0.022	0.034	0.057	0.008	0.000	0.001	0.012	0.030	0.029	0.029	0.003	0.006	0.105		0.514
SNP14	rs254664	0.010	0.034	0.000	0.184	0.262	0.023	0.000	0.116	0.035	0.384	0.386	0.381	0.957	0.029	0.297	0.003	

Upper diagonal figures are D' and lower diagonal figures are r^2

Pairwise LD mesurement using the online software SHEsis

Pairs in LD ($D' > 0.8$ or $r^2 > 0.8$) are shown in italics

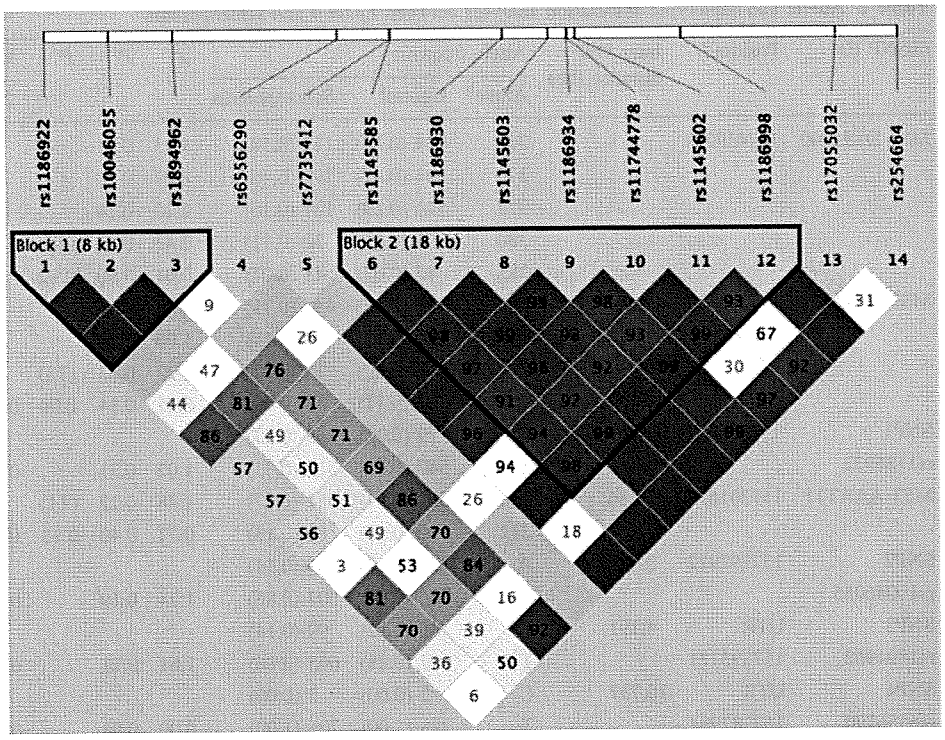

Fig. 2 Linkage disequilibrium (*LD*) block structure estimated from 14 SNPs by using Haploview

Discussion

The purpose of this investigation was to replicate Pimm et al.'s (2005) study of *Epsin 4* and its association with schizophrenia in a Japanese population. Pimm et al. (2005) originally reported that two SNPs, rs254664 (intron 1) and rs10046055 (5′ upstream region), in addition to two microsatellite markers D5S1403 (5′ flanking region) and AAAT11 (5′ upstream region), may be involved in the susceptibility to schizophrenia in the English, Irish, Welsh, and Scottish populations. In the present study, we examined a total of 17 polymorphisms and obtained weak evidence for an association at microsatellite 2 (D5S1403). However, when multiple testing was taken into consideration, the finding no longer reached significance. In addition, the risk-conferring allele was inconsistent between the two studies; we found an enrichment of the 203rd allele of microsatellite 2 in schizophrenics, whereas Pimm et al. (2005) found an enrichment of the same allele in controls. Furthermore, in the haplotype-based analysis, we did not find any evidence for an association.

Thus, we failed to replicate the widespread and highly significant associations with schizophrenia across 200,000 bp of *Epsin 4* reported by Pimm et al. (2005). Though we examined this region thoroughly by using 17 markers, we obtained no evidence for an association. The discrepancy found between Pimm et al.'s (2005) study and ours could result from a combination of factors. The first possibility may be due to the putative difference in allele frequencies between the English, Irish, Welsh, and Scottish population versus the Japanese population. It has been suggested that variations between ethnicities and populations in allele frequencies can dramatically affect the power to detect marginal differences (Marchini et al. 2005). According to the data bank of the International HapMap project (http://www.hapmap.org/index.html.en/), the allele frequency variation for rs10046055 and rs254664 is noticeable. Therefore, ethnic diversity of allele frequency of the SNPs may be a reason for negative findings in our present study. It is possible that there is a potential risk locus that is in strong LD with the investigated genetic variants in Pimm et al.'s (2005) population that is in weak or no LD with the variants in our population. However, to address this issue, we analyzed ten additional SNPs to thoroughly cover the entire genomic region of interest according to the HapMap database for the Japanese population; therefore, such a possibility is unlikely. Another possibility is that our negative results may be a type II error due to the potentially inadequate sample size. Our sample size (354 patients and 365 controls) had a power of 90% to detect an odds ratio of 1.9 or more if the T/A haplotype of HapB was assumed to be a risk (see Table 3). Incidentally, this haplotype gave rise to the most significant result ($P = 0.0005$) in the study of Pimm et al. (2005). This relatively weak power is due to the low frequency (0.06) of the T/A haplotype in our Japanese controls. If the A allele

Table 2 Allelic and haplotypic association analyses between *Epsin 4* and schizophrenia

dbSNP ID	Position[a]	Inter-SNP distance (bp)	Allele frequency			χ^2	P^c	P^d	Haplotype P (global)[e]			
			Allele[b]	Control	Schizophrenia				2 Locus	3 Locus	4 Locus	5 Locus
MS1 D5S1400	157439237	–	368	104 (0.16)	115 (0.18)	1.03	0.31					
			371	304 (0.47)	292 (0.46)	0.07	0.80					
			377	124 (0.19)	101 (0.16)	2.20	0.14					
			380	55 (0.08)	70 (0.11)	2.43	0.12	(T3)				
			383	45 (0.07)	45 (0.07)	0.01	0.90	0.12				
MS2 D5S1403	157346617	92,620	203	417 (0.66)	444 (0.71)	4.26	0.04					
			207	114 (0.18)	99 (0.16)	1.00	0.32					
			211	18 (0.03)	15 (0.02)	0.23	0.63	(T3)				
			215	67 (0.11)	56 (0.09)	0.91	0.34	0.04	0.64			
SNP1	157302083	44,534	A	179 (0.25)	189 (0.27)							
rs1186922			T	547 (0.75)	511 (0.73)	1.02	0.31		0.23	0.43		
MS3 AAAT11	157301697	386	248	567 (0.84)	562 (0.87)	1.48	0.22	(T1)				
			252	100 (0.15)	86 (0.13)	0.71	0.40	0.13	0.47	0.58	0.34	
SNP2	157297912	3,785	A	120 (0.17)	99 (0.14)							
rs10046055			T	606 (0.83)	607 (0.86)	1.74	0.19		0.23	0.43	0.77	0.31
SNP3	UTR	4,191	C	86 (0.12)	99 (0.14)							
rs1894962	157293721		T	642 (0.88)	605 (0.86)	1.61	0.21		0.23	0.26	0.47	0.77
SNP4	UTR	10,737	C	2 (0.00)	2 (0.00)							
rs6556290	157282984		T	726 (1.00)	706 (1.00)	0.00	0.98		0.22	0.23	0.27	0.47
SNP5	UTR	3,395	C	14 (0.02)	16 (0.02)							
rs7735412	157279589		T	712 (0.98)	690 (0.98)	0.20	0.66		1.00	0.21	0.23	0.26
SNP6	UTR	42	C	608 (0.84)	589 (0.83)							
rs1145585	157279547		T	116 (0.16)	117 (0.17)	0.08	0.78		0.76	0.84	0.43	0.38
SNP7	UTR	7,239	T	494 (0.68)	482 (0.68)							
rs1186930	157272308		C	232 (0.32)	222 (0.32)	0.03	0.86		0.78	0.67	0.67	0.52
SNP8	UTR	2,984	C	496 (0.68)	484 (0.69)							
rs1145603	157269324		T	230 (0.32)	222 (0.31)	0.01	0.92		0.92	0.74	0.64	0.63
SNP9	UTR	1,215	A	229 (0.32)	217 (0.31)							
rs1186934	157268109		G	491 (0.68)	479 (0.69)	0.06	0.80		0.89	0.89	0.68	0.57
SNP10	UTR	42	A	114 (0.16)	102 (0.14)							
rs11744778	157268067		G	608 (0.84)	604 (0.86)	0.50	0.48		0.78	0.75	0.75	0.72
SNP11	UTR	517	A	117 (0.16)	115 (0.16)							
rs1145602	157267550		G	609 (0.84)	587 (0.84)	0.02	0.89		0.78	0.58	0.56	0.56
SNP12	Intron 1	6,895	C	279 (0.38)	258 (0.37)							
rs1186998	157260655		G	447 (0.62)	448 (0.63)	0.54	0.46		0.23	0.30	0.42	0.54
SNP13	Intron 1	10,039	A	49 (0.07)	37 (0.05)							
rs17055032	157250616		C	679 (0.93)	671 (0.95)	1.44	0.23		0.39	0.36	0.54	0.51
SNP14	Intron 1	4,036	C	611 (0.84)	603 (0.85)							
rs254664	157246580		T	113 (0.16)	103 (0.15)	0.29	0.59		0.47	0.51	0.48	0.51

[a] Chromosome position was obtained from the HapMap database

[b] Microsatellites alleles presented by PCR fragment size (bp) with frequencies >0.03 in controls in either study

[c] P from 2 × 2 χ^2, with one *df*

[d] P from CLUMP Monte Carlo

[e] P from COCAPHASE-EM, -droprare 0.03 options

Table 3 Replication study of haplotype-based analysis between *Epsin 4* and schizophrenia

Groupe	Markers and haplotype			Case freq.	Cont freq.	*P* value[a]	
						Individual	Global
HapA	rs254664	rs10046055					
	C	A		0.05	0.08	0.098	
	T	A		0.09	0.09	0.743	
	C	T		0.80	0.77	0.151	
	T	T		0.06	0.07	0.664	0.341
HapB	rs1186930	rs10046055					
	C	A		0.10	0.10	0.563	
	T	A		0.04	0.06	0.133	
	C	T		0.22	0.22	0.835	
	T	T		0.64	0.62	0.418	0.471
HapC	rs10046055	AAAT11					
	T	248		0.85	0.82	0.189	
	A	252		0.12	0.14	0.249	0.234
HapD	rs254664	rs10046055	AAAT11				
	G	T	248	0.80	0.76	0.10	
	G	T	248	0.06	0.07	0.42	
	T	A	252	0.05	0.06	0.25	
	T	A	252	0.07	0.08	0.58	0.429
HapE	rs1145603	rs10046055	AAAT11				
	C	T	248	0.64	0.61	0.292	
	T	T	248	0.21	0.22	0.969	
	C	A	252	0.04	0.06	0.131	
	T	A	252	0.08	0.08	0.698	0.440
HapF	rs1145603	rs254664					
	C	C		0.69	0.68	0.940	
	T	C		0.17	0.16	0.671	
	T	T		0.15	0.16	0.591	0.819

[a] *P* from COCAPHASE-EM, -droprare 0.03 options

of rs10046055, which gave rise to the second significant result ($P = 0.002$) by Pimm et al. (2005), was assumed to be a risk, our sample size had a power of 90% to detect an odds ratio of 1.6 or more. Thus our sample size may have been limited by the inadequacy to detect a potentially weak effect of *Epsin 4* in Japanese. It is also possible that there may be a differential effect of *Epsin 4* on the development of schizophrenia between ethnic groups due to unknown factors. Alternatively, the positive association between schizophrenia and *Epsin 4* reported by Pimm et al. (2005) may have arisen by chance.

Subsequent to Pimm et al.'s (2005) investigation, two replication studies have been reported. Tang et al. (2006) reported a significant association of schizophrenia with some haplotypes of *Epsin 4* (252/T composed of AAAT11 and rs10046055, global $P = 0.0021$; T/T of rs1145603 and rs254664, $P = 0.0033$) in Han Chinese family trios, providing evidence for an association between schizophrenia and *Epsin 4*. The other replication study of Liou et al. (2006) found some evidence for an association, discovering a significant difference in allele frequency at rs1186922 ($P = 0.038$) and a significant difference in the frequency of a haplotype composed of two markers (rs1186922 and rs10046055). Liou et al. (2006) conservatively concluded that these findings occurred by multiple testing and chance and that their data did not support an association between schizophrenia and *Epsin 4*. However, Gurling et al. (2007) argued that the data of Liou et al. (2006) should be interpreted as supportive evidence for association. Since these two studies and ours were performed in Asian populations, if the association really exists, the obtained results should be similar. However, we obtained no evidence supporting the positive findings of Tang et al. (2006) or Liou et al. (2006).

In conclusion, we failed to identify a significant association between genetic variations of *Epsin 4* and schizophrenia in a Japanese population, suggesting that the examined region (5′ side) of *Epsin 4* does not have a major influence on susceptibility to schizophrenia in Japanese. Given the inconsistent findings across studies, more studies

should be conducted within different ethnic populations to draw more concrete conclusions. A meta-analysis of such studies and a multicenter study with a very large sample size will eventually be required. Since we examined only the 5′ side of the gene, the possibility remains that other unknown polymorphisms linked to the 3′ region of the *Epsin 4* gene may be associated with schizophrenia.

Acknowledgments We thank all patients for their participation in this study. This study was supported by the Japan Society for Promotion of Science (M. R.), Health and Labor Sciences Research Grants (Research on psychiatric and neurological diseases and mental health) (H. K.), Japan Health Sciences Foundation (Research on health sciences focusing on drug innovation) (H. K.), Grant-in-Aid for Scientific Research on Priority Areas of Applied Genomics from the Ministry of Education, Culture, Sports, Science and Technology of Japan (H. K.).

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Journal of Psychiatric Research 42 (2008) 425–432
JOURNAL OF PSYCHIATRIC RESEARCH
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TGFBR2 gene expression and genetic association with schizophrenia

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Received 12 October 2006; received in revised form 7 March 2007; accepted 18 April 2007

Abstract

TGFBR2 gene is a tumor suppressor gene located at chromosome 3p22, and the locus is reported to be linked with schizophrenia susceptibility. According to the previous studies, a reduced incidence of cancer is observed in schizophrenic patients compared with the general population and tumor suppressor genes may be associated with schizophrenia. We measured the mRNA expression of TGFBR2 gene in the peripheral leukocytes from 19 medication-free schizophrenics and 25 medication-free major depressive patients compared with age- and sex-matched control subjects using a quantitative real-time PCR method. We also followed up the TGFBR2 mRNA expression levels from 13 schizophrenics after several weeks – antipsychotic treatments. The TGFBR2 mRNA levels of medication free schizophrenics were significantly higher than those of control subjects and decreased to almost the same level as controls after antipsychotic treatment. On the other hand, the TGFBR2 mRNA levels of medication-free major depressive patients were not significantly different from controls. In genetic studies, we failed to find any association between the TGFBR2 gene and schizophrenia with 10 SNPs of TGFBR2 gene in Japanese subjects (279 subjects each) and there was no significant difference with haplotype analysis, either. Our results suggest that the TGFBR2 gene itself does not link to schizophrenia but that the TGFBR2 mRNA levels in the peripheral leukocytes may be a potential state marker for schizophrenia.

Keywords: TGFBR2; Gene expression; Leukocytes; Association analysis; Schizophrenia

1. Introduction

Schizophrenia is a complex psychiatric disorder that afflicts approximately 1% of the population throughout the world and has high heritability (Craddock et al., 2005). According to the previous studies, a reduced incidence of cancer is observed in schizophrenic patients compared with the general population (Catts and Catts, 2000; Grinshpoon et al., 2005). The possibility is explored to understand that alteration of the expression of oncogenes and/or tumor suppressor genes may account for tumor resistance associated with schizophrenia. Cui et al. reported that the tumor suppressor adenomatous polyposis coli (APC), which is involved in cell adhesion, was associated with schizophrenia and its expression levels were significantly increased in the leukocytes of schizophrenics no

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doi:10.1016/j.jpsychires.2007.04.002

matter how taking or not taking antipsychotic medications (Cui et al., 2005). There are several studies that the tumor suppressor gene p53 (TP53), which is a key element in maintaining genomic stability and cell apoptosis, is associated with schizophrenia (Yang et al., 2004; Ni et al., 2005).

Transforming growth factor-β receptor 2 (TGFBR2) gene is a putative tumor suppressor gene implicated in several malignancies (e.g. colon cancer, gastric cancer, gliomas, etc.) (Markowitz et al., 1995; Myeroff et al., 1995; Izumoto et al., 1997), and recently has been to be associated with Marfan syndrome (Mizuguchi et al., 2004). There have been several reports of Marfan syndrome cosegregating with schizophrenia within families (Romano and Linares, 1987; Sirota et al., 1990), which suggest that some genetic resemblances may be shared between schizophrenia and Marfan syndrome. The TGFBR2 gene consists of seven exons and encodes the human TGF-β receptor, type II. This receptor belongs to the serine-threonine kinase family of cell surface receptors, which regulates several cellular processes, including proliferation, cell cycle arrest, apoptosis, differentiation and formation of extra cellular matrix (Annes et al., 2003; ten Dijke and Hill, 2004). TGFBR2 is expressed in the brain as well as other tissues and its locus lies at chromosome 3p22, which has been previously reported to be linked with schizophrenia (Lewis et al., 2003). These above findings imply that TGFBR2 gene may be involved in the pathogenesis of schizophrenia.

To investigate the pathological role of TGFBR2 gene to schizophrenia, we measured the TGFBR2 mRNA expression levels in the peripheral leukocytes of medication-free 19 schizophrenic patients, 25 major depressive patients and age- and sex-matched control subjects using a quantitative real time PCR method. In addition, we conducted a genetic case-control study of the TGFBR2 gene with schizophrenia in Japanese subjects (schizophrenics; $n = 279$, control subjects; $n = 279$).

2. Materials and methods

2.1. Subjects for analysis

All patients and control subjects were biologically unrelated Japanese. The diagnosis of schizophrenia and major depression was made by at least two experienced psychiatrists according to DSM-IV criteria (American Psychiatric Association, 1994). Clinical symptoms were evaluated by the Brief Psychiatric Rating Scale scores (BPRS) (Overall and Gorham, 1962) in schizophrenic patients when blood samples were taken. Age- and sex-matched controls were in good physical health without a history of any psychiatric or serious somatic diseases and taking any medication during the sample collection period. Probands who had first-degree relatives with psychiatric disorders were excluded from the control subjects.

Table 1a
Demographic data for medication-free schizophrenic patients studied in TGFBR2 mRNA expression analysis ($N = 19$)

	Age (y.o)	Gender	Age at onset (years)	BPRS score	Family history of Schizophrenia in first-degree relative
S1	25	M	22	64	+
S2	24	M	24	42	–
S3	24	M	24	31	–
S4	27	M	24	37	–
S5	36	M	36	34	–
S6	39	M	38	59	–
S7	27	M	26	58	–
S8	20	F	19	46	–
S9	23	F	23	48	–
S10	34	F	31	36	–
S11	47	F	47	30	–
S12	15	F	13	30	+
S13	26	F	21	100	–
S14	23	M	23	31	–
S15	28	M	25	63	–
S16	47	F	47	37	–
S17	37	F	21	36	–
S18	30	F	25	41	–
S19	45	F	43	36	+

The age (years old: y.o) represent the age of the subject when the leukocytes were drawn. M = male, F = female, + indicates that at least one of the first-degree relatives has schizophrenia.

For the measurement of expression levels of the TGFBR2 mRNA, the subjects consisted of 19 medication-free patients with schizophrenia (subject number S1–S19, Tables 1a and 1b) (14 first-episode and drug-naïve schizophrenic patients, 5 schizophrenic patients without antipsychotic treatment for at least two months; 9 males and 10 females, mean age: 30.4 ± 9.3), 19 age- and sex-matched controls for schizophrenic patients (9 males and 10 females, mean age: 30.6 ± 8.6), 25 medication-free patients with major depression (17 first-episode and drug-naïve depressive patients, 8 depressive patients without antidepressant treatment for at least two months; 9 males and 16 females, mean age: 39.8 ± 13.2) and 25 age- and sex-matched controls for depressive patients (9 males and 16 females, mean age: 40.9 ± 13.1). In addition, The TGFBR2 mRNA levels after antipsychotic treatment for several weeks were investigated in 13 out of 19 subjects (subject number S1–S13, Tables 1a and 1b, 7 males and 6 females, mean age: 28.2 ± 8.6) who were able to be followed up and compared with 13 age- and sex-matched controls (7 males and 6 females, mean age: 28.6 ± 7.5).

For the genetic studies, we used genomic DNA samples from 279 in-patients (189 male and 90 female; mean age: 51.3 ± 13.7 years) with schizophrenia from eleven psychiatric hospitals in the neighboring area of Tokushima Prefecture in Japan (population: about 820,000). Age- and sex-matched controls were selected from volunteers after assessing psychiatric problems (189 male and 90 female; mean age: 51.4 ± 12.0) for the association and haplotype-based case-control studies.