

## 研究成果の刊行に関する一覧表

### 雑誌 (主なもの)

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

Yoshinori Saeki · Takashi Watanabe · Mikito Ueda · Atsushi Saito · Kazufumi Akiyama · Yoshimasa Inoue · Genta Hirokane · Sachiyo Morita · Naoto Yamada · Kazutaka Shimoda

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### 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 (%) =  $68.5 - 1.2 \times [\text{plasma concentration of PAX (ng/ml)}] - 33.0 \times (L/S=1, S/S=0) - 21.8 \times (\text{with comorbid physical illness}=1, \text{without comorbid physical illness}=0)$ .

**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

Y. Saeki · T. Watanabe · M. Ueda · K. Shimoda (✉)  
Department of Psychiatry,  
Dokkyo Medical University School of Medicine,  
880 Kitakobayashi, Mibu-machi, Shimotsuga,  
Tochigi 321-0293, Japan  
e-mail: shimoda@dokkyomed.ac.jp

A. Saito · K. Akiyama  
Department of Biological Psychiatry and Neuroscience,  
Dokkyo Medical University School of Medicine,  
880 Kitakobayashi, Mibu, Shimotsuga,  
Tochigi 321-0293, Japan

Y. Inoue  
MP Technopharma,  
955 Koiwai, Yoshitomicho, Chikujō,  
Fukuoka 871-8550, Japan

G. Hirokane · S. Morita · N. Yamada  
Department of Psychiatry, Shiga University of Medical Science,  
Seta Tsukinowacho, Otsu,  
Shiga 520-2192, Japan

### 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 =  $42.3 - 0.9 \times [\text{plasma concentration of PAX (ng/ml)}]$  [ $R=0.529$ ,  $P=0.014$ , 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 [ $^{11}\text{C}$ ](N,N-dimethyl-2-(2-amino-4-cyanophenylthio) benzylamine ([ $^{11}\text{C}$ ]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 $\pm$ SD=34.3 $\pm$ 9.8 years). Body weight ranged from 40 to 95 kg (57.3 $\pm$ 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 ( $\leq 2.0$  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  $-80^{\circ}\text{C}$  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)

**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

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.

### 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  $\mu\text{l}$  consisting of 20 ng of genomic DNA, 0.8 mM dNTP mixture, 0.05  $\mu\text{g}$  of sense and antisense primers (i.e., LPR5 and LPR3), 1 $\times$ PCR buffer, 1.5 mM  $\text{MgCl}_2$ , 5% dimethyl sulfoxide, and 0.5 U of AmpliTaq DNA polymerase (Applied Biosystems Japan, Tokyo, Japan). Annealing was performed at  $60^{\circ}\text{C}$  for 30 s, extension at  $72^{\circ}\text{C}$  for 1 min, and denaturation at  $94^{\circ}\text{C}$  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

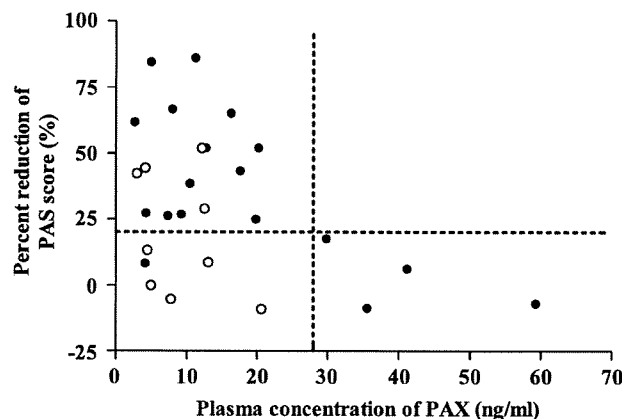
The mean PAS score was 21.6±6.9 (range, 9–34) at baseline before the initiation of pharmacotherapy with PAX. After 2 weeks of treatment, the mean PAS score improved to 15.1±6.9 (2–31). The mean percent reduction in PAS score (%) [percent reduction in PAS score = ((PAS score at baseline – PAS score 2 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 bromizolam 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 (%) =  $68.5 - 1.2 \times [\text{plasma concentration of PAX (ng/ml)}] - 33.0 \times (\text{L/S}=1, \text{S/S}=0) - 21.8 \times (\text{with comorbid physical illness}=1, \text{without comorbid physical illness}=0)$  (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 (%) =  $68.5 - 1.2 \times [\text{plasma concentration of PAX (ng/ml)}] - 33.0 \times (\text{L/S}=1, \text{S/S}=0) - 21.8 \times (\text{with comorbid physical illness}=1, \text{without comorbid physical illness}=0)$  [ $R=0.724$ ,  $P=0.001$ , 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<sub>1A</sub> 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<sub>1A</sub> 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<sub>1A</sub> 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<sub>1A</sub> receptors and for inhibiting PD symptoms; however, the increase in 5-HT in the somatodendritic area before the down-regulation of 5-HT<sub>1A</sub> 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<sub>1A</sub> 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<sub>1A</sub> receptors, which are autoreceptors located on cell bodies and dendrites, in drug naïve patients.

David et al. used <sup>11</sup>C-N-(2-(4-(2-methoxyphenyl)-1-piperazinyl)ethyl)-N(2-pyridyl)cyclohexanecarboxamide (<sup>11</sup>C]-WAY) as a selective radioligand for the 5-HT<sub>1A</sub> receptor and reported that the 5-HT<sub>1A</sub> receptor genotype showed no significant effects on 5-HT<sub>1A</sub> receptor binding in a PET study. On the other hand, 5-HT<sub>1A</sub> 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<sub>1A</sub> 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.

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# Association of the *HSPG2* Gene with Neuroleptic-Induced Tardive Dyskinesia

Aoi Syu<sup>1,2</sup>, Hiroki Ishiguro<sup>1,2</sup>, Toshiya Inada<sup>3</sup>, Yasue Horiuchi<sup>1,2</sup>, Syunsuke Tanaka<sup>1</sup>, Maya Ishikawa<sup>1</sup>, Makoto Arai<sup>4</sup>, Masanari Itokawa<sup>4</sup>, Kazuhiro Niizato<sup>5</sup>, Shuji Iritani<sup>5</sup>, Norio Ozaki<sup>6</sup>, Makoto Takahashi<sup>7</sup>, Akiyoshi Kakita<sup>8</sup>, Hitoshi Takahashi<sup>8</sup>, Hiroyuki Nawa<sup>8</sup>, Kazuko Keino-Masu<sup>9</sup>, Eri Arikawa-Hirasawa<sup>10</sup> and Tadao Arinami<sup>\*,1,2</sup>

<sup>1</sup>Department of Medical Genetics and Department of Molecular Neurobiology, Graduate School of Comprehensive Human Sciences, University of Tsukuba, Tsukuba, Ibaraki, Japan; <sup>2</sup>CREST, Japan Science and Technology Agency, Kawaguchi-shi, Saitama, Japan; <sup>3</sup>Institute of Neuropsychiatry, Seiwa Hospital, Tokyo, Japan; <sup>4</sup>Department of Schizophrenia Research, Tokyo Institute of Psychiatry, Tokyo, Japan; <sup>5</sup>Department of Psychiatry, Tokyo Metropolitan Matsuzawa Hospital, Tokyo, Japan; <sup>6</sup>Department of Psychiatry, School of Medicine, Nagoya University, Nagoya, Aichi, Japan; <sup>7</sup>Department of Psychiatry, Niigata University Graduate School of Medical and Dental Sciences, Niigata, Japan; <sup>8</sup>Brain Research Institute, Niigata University, Niigata, Japan; <sup>9</sup>Department of Molecular Neurobiology, Graduate School of Comprehensive Human Sciences, University of Tsukuba, Tsukuba, Ibaraki, Japan; <sup>10</sup>Research Institute for Diseases of Old Age, Department of Neurology, Juntendo University School of Medicine, Tokyo, Japan

Tardive dyskinesia (TD) is characterized by repetitive, involuntary, and purposeless movements that develop in patients treated with long-term dopaminergic antagonists, usually antipsychotics. By a genome-wide association screening of TD in 50 Japanese schizophrenia patients with treatment-resistant TD and 50 Japanese schizophrenia patients without TD (non-TD group) and subsequent confirmation in independent samples of 36 treatment-resistant TD and 136 non-TD subjects, we identified association of a single nucleotide polymorphism, rs2445142, (allelic  $p = 2 \times 10^{-5}$ ) in the *HSPG2* (heparan sulfate proteoglycan 2, perlecan) gene with TD. The risk allele was significantly associated with higher expression of *HSPG2* in postmortem human prefrontal brain ( $p < 0.01$ ). Administration of daily injection of haloperidol (HDL) for 50 weeks significantly reduced *Hspg2* expression in mouse brains ( $p < 0.001$ ). Vacuous chewing movements (VCMs) induced by 7-week injection of haloperidol–reserpine were significantly infrequent in adult *Hspg2* hetero-knockout mice compared with wild-type littermates ( $p < 0.001$ ). Treatment by the acetylcholinesterase inhibitor, physostigmine, was significantly effective for reduction of VCMs in wild-type mice but not in *Hspg2* hetero-knockout mice. These findings suggest that the *HSPG2* gene is involved in neuroleptic-induced TD and higher expression of *HSPG2*, probably even after antipsychotic treatment, and may be associated with TD susceptibility.

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**Keywords:** acetylcholine; neurogenetics; schizophrenia/antipsychotics; pharmacogenetics/pharmacogenomics; tardive dyskinesia

## INTRODUCTION

Antipsychotic-induced tardive dyskinesia (TD) is an involuntary movement disorder that develops in patients who are undergoing long-term treatment with antipsychotic medications. The clinical symptoms most commonly involve orobuccal, lingual, and facial muscles, especially in older individuals. The lingual involvement in the form of fine vermicular movements of the tongue while it is sitting

at the base of the oral cavity is a common early feature (Sachdev, 2000). In more severe cases, the movements may involve trunk and limbs (Tarsy and Baldessarini, 2006). Such movements lower the quality of life (QOL) of patients (Gerlach, 2002). Therefore, predicting those patients who are vulnerable to TD remains a high priority for psychiatrists in selecting the best medication for a given individual. Introduction of second-generation atypical antipsychotics has reduced the occurrence of TD to approximately 1% annually compared with the 5% frequency with typical agents (de Leon, 2007; Remington, 2007). Owing to the lack of effective treatments for TD, however, therapeutic management of TD can be problematic for schizophrenia patients receiving antipsychotic medications, especially for those patients who develop severe treatment-resistant TD. Therefore, the strategies to prevent TD are often discussed

\*Correspondence: Professor T Arinami, Department of Medical Genetics, Graduate School of Comprehensive Human Sciences, University of Tsukuba, 1-1-1 Tennoudai, Tsukuba, Ibaraki 305-8575, Japan, Tel: +81-29-853-3177, Fax: +81-29-853-3333, E-mail: tarinami@md.tsukuba.ac.jp  
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in the context of safety and use of antipsychotic drugs (Inada *et al*, 2008).

The etiology of TD is complex and remains unclear. Age, gender, and ethnicity are all the suggested risk factors for TD. Smoking, drinking, and using street drugs may also increase the risk of TD (Menza *et al*, 1991). There is some evidence for a genetic component to TD (Muller *et al*, 2004) and molecular genetic studies of TD were conducted to identify genes related to TD (Malhotra *et al*, 2004).

The pathophysiology of TD is not completely understood. The causative role of antipsychotic and other dopamine antagonists resulted in the proposal of the dopamine supersensitivity hypothesis of TD (Klawans *et al*, 1980). However, as the hypothesis explains only some aspects of TD, many other pathophysiological models including changes in other neurotransmitter signaling systems that are affected by neuroleptics have been considered. They include gamma-aminobutyric acid (Gerlach and Casey, 1988), norepinephrine (Saito *et al*, 1986), serotonin (Haleem, 2006), and acetylcholine (ACh) (Tammenmaa *et al*, 2002).

The advent of single nucleotide polymorphism (SNP) chips for genome-wide association analysis has made screening of susceptibility genes for TD possible. We carried out a genome-wide association study of treatment-resistant TD in schizophrenia patients and reported that SNPs associated with TD were aggregated significantly in genes belonging to the gamma-aminobutyric acid receptor signaling pathway (Inada *et al*, 2008). In this study, we analyzed the *HSPG2* gene, which includes SNPs that showed the most significant association with TD in our genome-wide association study.

## MATERIAL AND METHODS

### Ethical Considerations

This study was initiated after approval by the ethics committee of each institution. Written informed consent was obtained from all patients after adequate explanation of the study.

### Human Subjects

Human subjects in this study were 86 Japanese schizophrenia patients with TD and 186 Japanese schizophrenia patients without TD, who have been described elsewhere (Inada *et al*, 2008). Briefly, subjects were identified at psychiatric hospitals located around Tokyo and Nagoya areas of Japan. All patients satisfied the diagnostic criteria of DSM-IV (Association, 1994) for schizophrenia. All subjects and their parents were of Japanese descent. All subjects had been receiving antipsychotic therapy for at least 1 year and their TD status was monitored for at least 1 year. TD was assessed according to the Japanese version of the Abnormal Involuntary Movement Scale. TD was diagnosed according to the criteria proposed by Schooler and Kane (Schooler and Kane, 1982). Once TD was identified, the patients were followed up and received standard therapeutic regimens for TD to minimize TD symptoms. If TD persisted even after 1 year of therapy, patients were considered potential treatment-resistant TD

patients. Treatment-resistant TD patients were defined as those patients with dyskinetic movements that persisted for more than 1 year and did not improve even after 1 year of appropriate treatment after guideline-recommended therapeutic regimens for TD. We hypothesized that treatment-resistant TD, a severe form of TD, was suitable for detection of genetic association with TD. Only treatment-resistant TD patients were included as those affected with TD in this study.

### Genotyping, Resequencing, and Statistics

Association screening was performed using the Illumina Sentrix Human-1 Genotyping 109k BeadChip according to the manufacturer's instructions (Illumina, San Diego CA, USA). All DNA samples were subjected to rigorous quality control to check for fragmentation and amplification. Approximately 750 ng of genomic DNA was used in each sample. Normalized bead intensity data obtained for each sample was entered into the Illumina BeadStudio 3.0 software, which converted fluorescence intensities into SNP genotypes. A GenCall Score of 0.85 was used as a minimum threshold for per-sample genotyping completeness. The mean call rate across all samples was 97%. After removing SNPs with a low genotyping rate ( $p < 0.95$ ;  $n = 3952$ ), SNPs deviating from the Hardy-Weinberg equilibrium ( $p < 0.001$ ;  $n = 135$ ), SNPs with low minor allele frequency (MAF  $< 0.05$ ;  $n = 2762$ ), and SNPs located outside exons and introns, we screened for SNPs associated with TD using 40 573 SNPs. SNPs located within 10 kb from the 5' and 3' ends of known genes were included. SNPs in the linkage disequilibrium (LD) of  $r^2 > 0.8$  with other SNPs were excluded. The call rate was at least 99.4% for the 40 573 SNPs. The concordance rate was evaluated by comparisons of genotypes in the 100 screening samples and this gave concordance of over 98% for each sample. Genotyping using TaqMan probes (Applied Biosystems, Foster City, CA, USA) was carried out twice for each SNP, and genotype concordance was 99.7%. Genotyping completeness was  $> 0.99$ . We treated these uncalled or discrepant genotypes as missing genotypes.

To screen for novel polymorphisms, we used direct sequencing with a Big Dye Terminator Cycle Sequencing kit and ABI PRISM 3100 Genetic Analyzer (Applied Biosystems, Foster City, CA, USA). All exons and the exon-intron junctions of the *HSPG2* gene were amplified from the genomic DNAs of the 86 TD group patients. The sequences of primers for mutation screening are available on request.

For a more detailed analysis of the associations between SNPs in the *HSPG2* gene and TD, the tag SNPs in the gene were selected using the Haploview program (<http://www.broad.mit.edu/mpg/haploview/>) with the condition of an  $r^2$  threshold of 0.8 and a minor allele frequency of 0.1, and genotyped by the TaqMan method. Allelic discrimination was performed using the ABI PRISM 7900HT Sequence Detection System using SDS 2.0 software (Applied Biosystems, Foster City, CA, USA).

Allelic associations between SNPs and TD, and departure from the Hardy-Weinberg equilibrium were evaluated by  $\chi^2$  test or Fisher's exact test. Bonferroni's correction for multiple comparisons was applied.

## Human Postmortem Brains

Brain specimens were from individuals of European (Australian) and Japanese descent. The Australian sample comprised 10 schizophrenic patients and 10 age- and gender-matched controls. The diagnosis of schizophrenia was made according to the Diagnostic and Statistical Manual of Mental Disorders (DSM)-IV criteria (American Psychiatric Association (1994)) by a psychiatrist and a senior psychologist. Control subjects had no known history of psychiatric illness. Tissue blocks were cut from gray matter in an area of the prefrontal cortex referred to as Brodmann's area 9 (BA9). Japanese samples of BA9 gray matter from Japanese brain specimens consisted of six schizophrenic patients and 11 age- and gender-matched controls. In addition, postmortem brains of 37 deceased Japanese patients with schizophrenia were also analyzed. The Japanese subjects met the DSM-III-R criteria for schizophrenia. Details of the condition of the postmortem brains have been described elsewhere (Ishiguro *et al*, 2008; Koga *et al*, 2009).

## Analysis of *Hspg2* Transcription in Human Brain Tissue

Total RNA was extracted from human brain tissues with ISOGEN Reagent (Nippon Gene, Tokyo, Japan). The RNA quality was checked using a Nanodrop ND-1000 spectrophotometer (LMS, Tokyo, Japan) to have an OD 260/280 ratio of 1.8–2 and an OD 260/230 of 1.8 or greater. Expression of the *HSPG2* genes was analyzed by the TaqMan real-time polymerase chain reaction system (Applied Biosystems, Foster City, CA). From RNA, cDNA was synthesized with Revertra Ace (Toyobo, Tokyo, Japan) and oligo dT primers. Expression of the *HSPG2* gene was analyzed with an ABI PRISM 7900 HT Sequence Detection System (Applied Biosystems), with the TaqMan gene expression assays for *HSPG2* (Hs01078535\_m1), and normalized to the expression of Human GAPDH Control Reagents (Applied Biosystems).

Genotype effects on *HSPG2* expression were analyzed in Australian subjects and replicated in Japanese subjects using analysis of variance followed by Tukey's *post hoc* tests by JMP software version 7.0.1 (SAS Institute, Cary, NC, USA) was used.

## Animals

Animals were same-sex housed before behavior testing. The same animals were used for all behavior tests.

Four-week-old C57BL/6J male mice (weight: 20–25 g) treated with haloperidol (HDL) or vehicle-saline and 7-week-old male mice (wild type: 8; *Hspg2*<sup>+/-</sup>: 7) with orofacial dyskinesia were housed under 10 h: 14 h light/dark conditions with normal food and water *ad libitum*, with mice housed separately in groups of 4 or 5 mice.

The generation of *Hspg2* knockout mice and the phenotypes of the mice have been described elsewhere (Arikawa-Hirasawa *et al*, 1999). As *Hspg2* null mice are embryonic lethal, timed matings between heterozygotes were carried out to generate homozygous and wild-type mice in this study.

All animal protocols were approved by the Animal Care and Use committee of University of Tsukuba.

## Drugs

Reserpine (methyl reserpate 3,4,5-trimethoxycinnamic acid ester; Wako, Osaka, Japan) and HDL (Wako, Osaka, Japan) were diluted in glacial acetic acid and then diluted in distilled water. Physostigmine (Wako), a reversible cholinesterase inhibitor, was diluted in saline. All solutions were treated subcutaneously in volumes not exceeding 10 ml/kg body weight.

## HDL Treatment

To examine the effects of antipsychotic treatments on gene expression, we made two groups: an acute treatment group: 4-week-old C57BL/6J male mice were treated with intraperitoneal injection (i.p.) of 1.0 mg/kg HDL ( $n=10$ ) or vehicle-saline ( $n=10$ ) once each day for 4 weeks; and a long-term treatment group: 4-week-old C57BL/6J male mice were treated with intraperitoneal injection of 1.0 mg/kg HDL ( $n=10$ ) or vehicle-saline ( $n=10$ ) once each day for 50 weeks. Mice were killed 4 h after the last injection to obtain brain tissues.

## Induction of Vacuous Chewing Movements

Mice were treated with i.p. of 2 mg/kg HDL and 0.3 mg/kg reserpine every day for 7 weeks to induce the putative TD analogue vacuous chewing movements (VCMs) (Araujo *et al*, 2004; Burger *et al*, 2005; Naidu *et al*, 2003). Before injection and 4 hours after the injection on the 47th day, locomotor activity test and rotarod test were carried out. On the 48th and 49th days, 1, 2, 3, 4, and 24 h after the last injection, the animals were observed for quantification of VCMs for 2 days. On the 50th day, to verify the effects of physostigmine on VCMs, mice were injected with 0.1 mg/kg physostigmine. At 1, 2, 3, 4, and 24 h after the injection of physostigmine, the animals were observed for quantification of VCMs. On the 51st and 52nd day, mice were treated with 2 mg/kg HDL and 0.3 mg/kg reserpine and then observed for quantification of VCMs. On the 53rd day, mice were treated with vehicle-saline, and 1, 2, 3, 4, and 24 h after the injection, the animals were observed for quantification of VCMs.

## Analysis of *Hspg2* Transcription in Brain Tissue of Mice

The prefrontal cortex, midbrain, hippocampus, thalamus, and striatum were taken by dissection, and total RNA was extracted with an RNeasy kit (Qiagen, K.K., Tokyo, Japan). After cDNA synthesis from total RNA samples, the transcription level of cDNA samples was analyzed by a TaqMan Expression assay for *Hspg2* (Mm00464581\_m1; Applied Biosystems) and normalized to that of rodent *Gapdh* with Rodent *Gapdh* Control Reagents (Applied Biosystems). The average relative expression levels of five regions were compared with the saline groups by Student's *t*-test.

**Table 1** Allelic *p*-Values of SNPs for Association with TD in Screening and Replication Samples

SNP	Chromosome	Location	Gene	Position relative to gene	Allele frequency			Uncorrected allelic <i>p</i>	
					TD group	Non-TD group	Screening	Replication	Combined
rs7529452	chr1	1p36.22	<i>PLOD1</i>	coding	0.396	0.381	<b>0.001</b>	<b>0.05</b>	
rs2445142	chr1	1p36.12	<i>HSPG2</i>	intron	0.579	0.380	<b>0.001</b>	<b>0.002</b>	<b>0.00002</b>
rs1934712	chr1	1p21.1	<i>COL11A1</i>	flanking_3UTR	0.435	0.343	<b>0.0007</b>	0.98	
rs2306444	chr1	1p12	<i>MAN1A2</i>	intron	0.482	0.392	<b>0.0005</b>	0.59	
rs869807	chr1	1p12	<i>TBX15</i>	flanking_3UTR	0.282	0.185	<b>0.0009</b>	0.97	
rs6668395	chr1	1q41	<i>DUSP10</i>	flanking_5UTR	0.418	0.535	<b>0.001</b>	0.59	
rs6426327	chr1	1q44	<i>SMYD3</i>	intron	0.412	0.324	<b>0.00002</b>	0.20	
rs4558632	chr2	2p21	<i>EML4</i>	flanking_5UTR	0.253	0.171	<b>0.0003</b>	0.82	
rs6714424	chr2	2p16.2	<i>ASB3</i>	flanking_5UTR	0.212	0.120	<b>0.0007</b>	0.76	
rs2060279	chr2	2p12	<i>LRRTM4</i>	flanking_5UTR	0.685	0.777	<b>0.0005</b>	0.52	
rs11694702	chr2	2q13	<i>BUB1</i>	flanking_5UTR	0.329	0.241	<b>0.001</b>	0.76	
rs1873201	chr2	2q24.3	<i>KCNH7</i>	flanking_5UTR	0.395	0.301	<b>0.002</b>	0.78	
rs11688866	chr2	2q31.3	<i>UBE2E3</i>	flanking_5UTR	0.452	0.581	<b>0.0005</b>	0.45	
rs3749279	chr3	3p22.3	<i>STAC</i>	intron	0.202	0.099	<b>0.001</b>	0.59	
rs6443468	chr3	3q26.32	<i>TBL1XR1</i>	flanking_5UTR	0.373	0.511	<b>0.001</b>	0.26	
rs13115988	chr4	4q22.1	<i>LOC285513</i>	3UTR	0.694	0.645	<b>0.001</b>	0.32	
rs700237	chr5	5p13.1	<i>C9</i>	flanking_5UTR	0.898	0.830	<b>0.0009</b>	0.80	
rs832582	chr5	5q11.2	<i>MGC33648</i>	flanking_5UTR	0.641	0.543	<b>0.001</b>	0.37	0.03
rs13153252	chr5	5q14.3	<i>EDIL3</i>	intron	0.375	0.486	<b>0.0002</b>	0.94	
rs6594324	chr5	5q21.3	<i>FER</i>	flanking_5UTR	0.692	0.578	<b>0.001</b>	0.37	
rs915125	chr6	6q14.1	<i>FAM46A</i>	flanking_5UTR	0.207	0.262	<b>0.0009</b>	0.41	
rs2691180	chr6	6q21	<i>CDC2L6</i>	intron	0.789	0.890	<b>0.0007</b>	0.41	
rs9376506	chr6	6q24.1	<i>CITED2</i>	flanking_5UTR	0.564	0.457	<b>0.001</b>	0.71	
rs1832445	chr6	6q24.1	<i>FLJ39824</i>	flanking_3UTR	0.494	0.604	<b>0.001</b>	0.94	
rs3735478	chr7	7p13	<i>DKFZp76112123</i>	coding	0.058	0.145	<b>0.001</b>	0.32	0.006
rs1047053	chr7	7q36.2	<i>DPP6</i>	3UTR	0.657	0.758	<b>0.0005</b>	0.3	0.02
rs2583086	chr8	8q13.2	<i>SULF1</i>	intron	0.169	0.255	<b>0.001</b>	0.95	
rs4738269	chr8	8q13.3	<i>KCNB2</i>	intron	0.571	0.396	<b>0.0007</b>	<b>0.04</b>	<b>0.0002</b>
rs2927111	chr8	8q23.1	<i>STARS</i>	flanking_5UTR	0.369	0.487	<b>0.0004</b>	0.4	0.01
rs3019982	chr8	8q23.1	<i>ANGPT1</i>	flanking_3UTR	0.612	0.479	<b>0.0002</b>	0.18	0.004
rs4242345	chr8	8q24.13	<i>ANXA13</i>	flanking_3UTR	0.659	0.764	<b>0.00004</b>	0.56	
rs1413299	chr9	9q22.33	<i>COL15A1</i>	intron	0.682	0.746	<b>0.001</b>	0.73	
rs2274359	chr10	10p15.3	<i>RBM17</i>	intron	0.929	0.834	<b>0.0005</b>	0.51	
rs1932596	chr10	10q21.1	<i>PCDH15</i>	intron	0.628	0.543	<b>0.0007</b>	0.51	
rs1058198	chr10	10q22.3	<i>DLG5</i>	coding	0.152	0.290	<b>0.0006</b>	0.22	0.0007
rs10748816	chr10	10q24.32	<i>ELOVL3</i>	intron	0.494	0.634	<b>0.00008</b>	0.13	0.002
rs2246775	chr10	10q24.32	<i>GBF1</i>	intron	0.646	0.747	<b>0.0009</b>	0.66	
rs765934	chr10	10q26.3	<i>MGMT</i>	flanking_5UTR	0.732	0.642	<b>0.0012</b>	0.74	
rs886292	chr11	11p15.1	<i>ABCC8</i>	intron	0.825	0.696	<b>0.0005</b>	<b>0.02</b>	0.0015
rs286925	chr11	11p13	<i>EHF</i>	5UTR	0.542	0.611	<b>0.0005</b>	0.75	
rs568758	chr11	11q13.4	<i>SPCS2</i>	intron	0.738	0.818	<b>0.0009</b>	0.82	
rs624786	chr11	11q13.4	<i>NEU3</i>	flanking_5UTR	0.735	0.812	<b>0.0015</b>	0.83	
rs1444590	chr12	12q13.11	<i>SLC38A1</i>	intron	0.789	0.839	<b>0.0005</b>	0.39	
rs1154664	chr12	12q24.32	<i>KIAA1906</i>	flanking_3UTR	0.688	0.590	<b>0.0002</b>	0.80	
rs1924174	chr13	13q33.3	<i>LIG4</i>	flanking_3UTR	0.282	0.195	<b>0.0013</b>	0.19	0.04
rs1189827	chr14	14q22.3	<i>SEC10L1</i>	flanking_3UTR	0.741	0.663	<b>0.0007</b>	0.45	
rs11625123	chr14	14q32.12	<i>ITPK1</i>	intron	0.124	0.225	<b>0.0009</b>	0.45	0.007
rs10140345	chr14	14q32.2	<i>VRK1</i>	flanking_3UTR	0.300	0.273	<b>0.0011</b>	0.09	



Table 1 Continued

SNP	Chromosome	Location	Gene	Position relative to gene	Allele frequency			Uncorrected allelic <i>p</i>	
					TD group	Non-TD group	Screening	Replication	Combined
rs2061051	chr15	15q12	GABRG3	intron	0.206	0.350	<b>0.0014</b>	<b>0.04</b>	<b>0.0006</b>
rs3764211	chr15	15q13.1	APBA2	flanking_3UTR	0.726	0.815	<b>0.0013</b>	0.12	0.005
rs1036673	chr15	15q24.1	PML	3UTR	0.721	0.592	<b>0.0006</b>	0.64	
rs3809729	chr17	17p12	DNAH9	flanking_5UTR	0.867	0.869	<b>0.0007</b>	0.11	
rs4630608	chr17	17p11.2	FBXW10	intron	0.250	0.274	<b>0.0010</b>	<b>0.04</b>	
rs2287352	chr17	17q12	ACACA	flanking_5UTR	0.247	0.306	<b>0.0014</b>	0.85	
rs3744165	chr17	17q25.3	FLJ13841	5UTR	0.093	0.130	<b>0.0010</b>	0.15	
rs474122	chr18	18p11.31	DLGAP1	flanking_5UTR	0.404	0.330	<b>0.0002</b>	0.38	
rs12460403	chr19	19p13.3	HMG20B	flanking_3UTR	0.285	0.194	<b>0.0011</b>	0.55	
rs437168	chr19	19q13.12	NPHS1	coding	0.223	0.139	<b>0.0007</b>	0.9	
rs10419669	chr19	19q13.31	CBLC	intron	0.094	0.179	<b>0.0003</b>	0.84	
rs8112223	chr19	19q13.41	HAS1	flanking_5UTR	0.314	0.219	<b>0.0003</b>	0.43	
rs2328500	chr20	20p11.23	C20orf26	intron	0.376	0.324	<b>0.001</b>	0.12	
rs7281019	chr21	21q22.11	TCP1OL	intron	0.924	0.862	<b>0.00008</b>	0.57	0.04
rs2056965	chr22	22q12.3	LOC91464	flanking_5UTR	0.422	0.348	<b>0.0002</b>	0.49	

Abbreviations: SNP, single nucleotide polymorphism; TD, tardive dyskinesia.  
*p*-Values with bold emphasis indicate *p* < 0.05 in 1st *p* and 2nd *p*, and combined *p* < 1st *p*.

### Evaluation of VCMs

Mice were placed individually in observation cages (16 × 17 × 19 cm<sup>3</sup>) without food. Hand-operated counters were used to quantify VCMs continuously for 5 min. VCMs were referred to as single mouth openings in the vertical plane not directed toward physical material. If VCMs occurred during a period of grooming, they were not taken into account. Mirrors were placed under the floor and behind the back wall of the cage to permit observation of oral movements when the animal faced away from the observer. The observations were made by two observers who were blind to the animal's group assignment. The observation criteria were not subjective, because an excellent inter-observer agreement was found in a previous pilot experiment (Pearson's correlation = 0.98). All behavioral experiments were conducted between 1000 and 1800 hours.

### Locomotor Activity

The locomotor activity test was conducted between 1200 and 1700 hours in a dimly lit testing room. Mice were habituated to the room for at least 30 min before testing. The locomotor activity test was videotaped with a Sony Digital Video Camera (Sony, Tokyo, Japan). The behavioral testing apparatus was a black Plexiglas rectangular box (41 cm long × 22 cm wide × 20.5 cm tall) and activity was recorded for 20 min. The total distance traveled (locomotion) was scored.

### Rotarod Test

The rotarod test was conducted between 1200 and 1700 hours in a dimly lit testing room. All mice were brought to

the testing room in their home cages and were allowed to sit undisturbed in the testing room for at least 5 min before the start of behavioral testing. Motor performance was assessed by rotarod (Med Associates, St Albans, VT). A 1-min training session was given to each mouse on the rotarod (diameter 8 cm, 7 rpm) 5 min before the first measurement. Motor performance (time until the first fall) was registered during a 2-min session.

### Statistical Analysis for Behavioral Data and Gene Expression in Animal Experiments

Effects of genotype, drug treatment, and time were analyzed using analysis of variance) followed by Tukey's *post hoc* tests or using Student's *t*-test. Individual differences of the number of VCMs between before and after injection of physostigmine and saline were tested by nonparametric test for one sample test of mean = 0.

## RESULTS

### Association Study

We screened for SNPs associated with TD using 40 573 tag SNPs on the Sentrix<sup>®</sup> Human-1 Genotyping BeadChip (Illumina) to identify loci associated with susceptibility to TD in 50 TD and 50 non-TD subjects (Inada *et al*, 2008). The potential impact of population structure on this association study was evaluated by using the genome-wide  $\chi^2$  inflation factor,  $\lambda$ , as a genomic control (Devlin and Roeder, 1999; Devlin *et al*, 2001). The estimated value of  $\lambda$  was 1.04, by which genome-wide association *p*-values were corrected. The lowest uncorrected allelic *p*-value for association with TD was  $1 \times 10^{-5}$ . Therefore, no SNP was

significantly associated with TD after Bonferroni correction. An attempt was made to replicate the association of 63 SNPs, which were allelic  $p$ -values  $<0.002$  and located within 10 kb from known genes with the TaqMan genotyping assay (Table 1). A potential association was found for four SNPs (allelic  $p < 0.05$ ) (Table 1). However, no significant association was found after correction for multiple testing of 63 SNPs in the replication sample only. Among these four SNPs, an association between GABRG3 SNP and TD has already been reported (Inada *et al*, 2008). The lowest allelic  $p$ -value for the association was found for rs2445142 ( $p = 2 \times 10^{-5}$ ) when the initial genome-wide sample and replication sample were combined. The SNP is located in the HSPG2 gene.

Next, we tested associations of 24 tag SNPs including rs2445142 in the HSPG2 gene with TD and found a nominal significant association for five SNPs in addition to rs2445142 (Table 2). Other than rs2445142, we found a significant association of rs2124368 located in intron 43 of the HSPG2 gene with TD even after applying Bonferroni's correction for multiple testing (uncorrected allelic  $p = 0.0003$ , corrected  $p = 0.007$ ). The SNP rs2124368 was not in LD with rs2445142, which was located in intron 1 ( $D' = 0.13$ ,  $r^2 = 0.01$ ).

Subsequently, we genotyped the SNPs of rs2501255 (intron 1), rs2501257 (intron 1), rs897474 (intron 3), rs2254357 (exon 6), rs2254358 (exon 6), and rs2497632 (intron 9), because of the expected complete LD between these SNPs and rs2445142 based on the HapMap database. We confirmed that they were in complete LD with rs2445142 ( $r^2 = 1.00$ ). These SNPs were located in introns 1-9 of the HSPG2 gene. Age, sex, and age of onset were not associated with these SNPs. Acute extrapyramidal symptoms were associated with rs2445142 and the six SNPs in complete LD with rs2445142 (allelic  $p = 0.00002$ ). Resequencing all exons of the HSPG2 gene in patients with TD did not reveal novel SNPs. Finally, we genotyped missense SNPs of rs3736360, rs2229493, rs2291827, rs2228349, rs2229491, rs2229490, rs2229489, rs2229475, rs897471, rs2229481, and rs989994, which were listed in dbSNP and did not find significant associations of these SNPs with TD (data not shown). Thus, we tested a total of 103 SNPs, including 41 SNPs in the HSPG2 gene, in our total subjects of 86 TD and 136 non-TD patients.

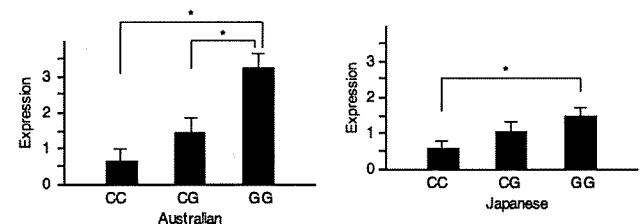
**Association Between Hspg2 Expression Levels in the Postmortem Prefrontal Cortex and Rs2445142**

The transcription level in the postmortem prefrontal cortex, as measured by TaqMan real-time polymerase chain reaction, was not significantly different by diagnosis, age, sex, postmortem intervals, or pH of brain samples. A significant genotype effect on HSPG2 gene expression levels was observed in 20 Australian subjects ( $F(2, 17) = 4.9$ ,  $p = 0.02$ ) and replicated in 54 Japanese subjects ( $F(2, 51) = 3.5$ ,  $p = 0.04$ ). The association was significant in the combined subjects ( $F(2, 71) = 7.6$ ,  $p = 0.001$ ). Tukey's *post hoc* tests showed that HSPG2 expression levels were significantly higher in the subjects with the GG genotype than in those with the CC genotype (Figure 1). Unfortunately, information about TD in the brains we analyzed was not available.

**Table 2** Allelic  $p$ -Values of Tag SNPs in the HSPG2 Gene for Association with TD

Location	Allele frequency*				Allelic $p$
	Allele	TD group	Non-TD group		
rs3736360	exon 96 (N4331S)	A/G	0.19	0.20	0.8715
rs3767137	intron 77	A/G	0.23	0.19	0.2759
rs10917053	intron 71	A/G	1.00	0.99	0.3308
rs37355045	intron 64	G/A	0.84	0.81	0.4235
rs2290501	intron 60	C/A	0.22	0.21	0.7134
rs1563370	intron 52	A/G	0.35	0.27	0.0687
rs2229475	exon 47 (I1967V)	G/A	0.01	0.01	0.9477
rs2305562	intron 43	A/G	0.61	0.49	0.0117
rs4654991	intron 42	G/A	0.39	0.36	0.5605
rs2124368	intron 42	G/A	0.77	0.60	0.0003
rs897472	intron 36	C/A	0.09	0.05	0.1098
rs897471	exon 36 (V1503A)	A/G	0.88	0.87	0.7005
rs2229478	exon 8 (L248L)	A/G	0.53	0.42	0.0273
rs3767141	intron 6	G/A	0.66	0.60	0.1811
rs2445142	intron 1	G/C	0.58	0.38	0.00002
rs878949	intron 1	A/G	0.22	0.20	0.5867
rs1545593	intron 1	C/A	0.41	0.30	0.0122
rs1002480	intron 1	G/C	0.41	0.32	0.0368
rs6698486	intron 1	G/A	0.46	0.38	0.0754
rs10799719	intron 1	G/A	0.80	0.75	0.1789
rs9426785	intron 1	A/G	0.57	0.55	0.7389
rs4654773	intron 1	A/G	0.45	0.45	0.9165
rs11587857	intron 1	G/A	0.50	0.46	0.3465
rs4233280	5' flanking	A/G	0.07	0.03	0.0588

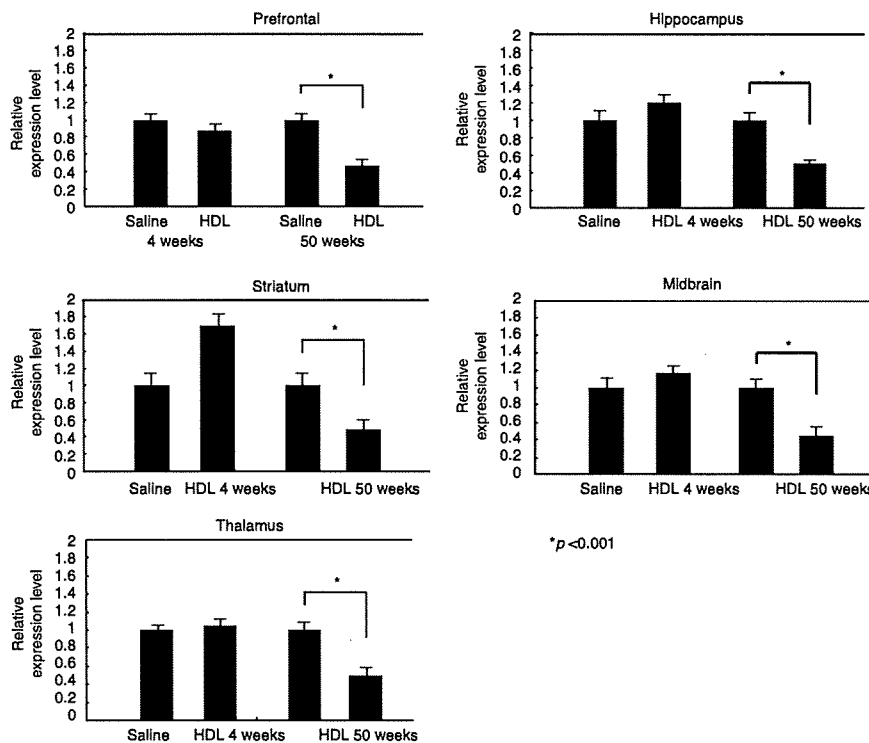
Abbreviations: SNP, single nucleotide polymorphism; TD, tardive dyskinesia  
\*The frequency of the first allele.



**Figure 1** HSPG2 expression levels in the postmortem prefrontal region by genotype. The vertical scores show the average (SEM) of relative expression levels in each of the three genotype groups, compared with the mean gene expression in the total samples. \*Indicates  $p < 0.05$  by Tukey's *post hoc* tests.

**Hspg2 Gene Expression in the Mouse Brains by HDL Treatment**

Hspg2 expression levels were evaluated in the mouse brain after treatment with the antipsychotic drug, HDL. The expression of Hspg2 levels did not alter after a 4-week treatment of HDL except for the striatum where Hspg2 was expressed significantly higher than after the saline



**Figure 2** Effects of haloperidol (HDL) on *HSPG2* gene expression in the mouse brains. Relative expression levels of *Hspg2* from the prefrontal cortex, midbrain, hippocampus, thalamus, and striatum in the mouse brains after treatment with HDL for 4 weeks ( $n = 10$ ) and HDL for 50 weeks ( $n = 10$ ) were compared with the saline groups for 4 weeks ( $n = 10$ ) and 10 weeks ( $n = 10$ ) by Student's *t*-test.

treatment. Significantly lower expression of *Hspg2* was observed in all brain regions after a 50-week treatment with HDL than after a 50-week treatment with saline (Figure 2) ( $F(1, 18) = 42.9$ ,  $p < 0.0001$  at the prefrontal cortex;  $F(1, 18) = 20.1$ ,  $p = 0.0003$  at the hippocampus;  $F(1, 18) = 15.9$ ,  $p = 0.0009$  at the striatum;  $F(1, 17) = 19.3$ ,  $p = 0.0004$  at the midbrain;  $F(1, 18) = 16.5$ ,  $p = 0.0007$  at the thalamus).

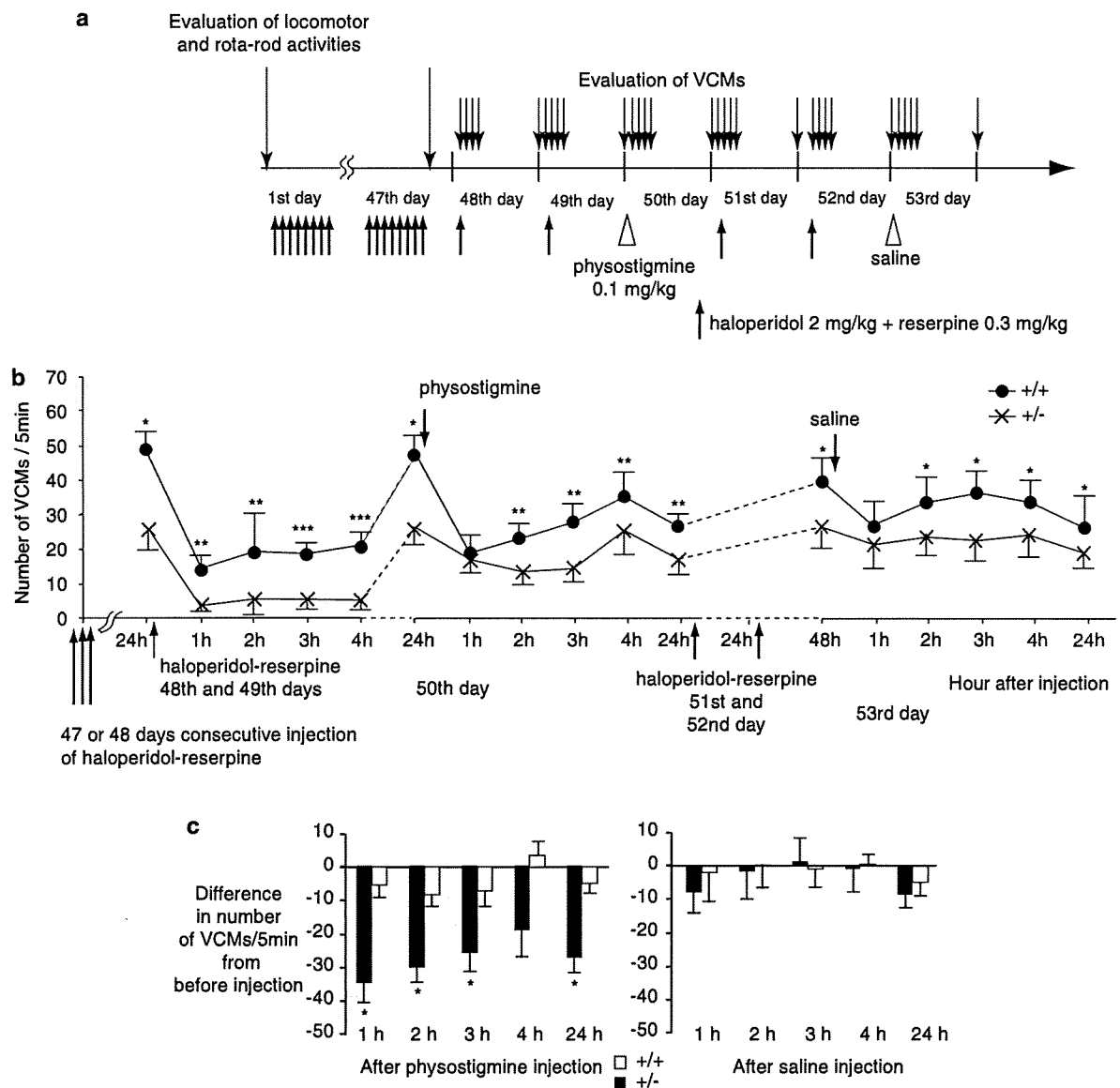
#### Analysis of VCMs Induced by Haloperidol-Reserpine in *Hspg2* Knockout Mice

As we could not induce VCMs by administration of HDL only to mice, VCMs induced by long-term treatment with HDL and reserpine in female *Hspg2* hetero-knockout mice and female wild-type gene litters were measured to evaluate the relationship between expression levels of *Hspg2* and TD (Figure 3a). *Hspg2*-null knockout mice were embryonic lethal. The relative expression levels of *Hspg2* in *Hspg2* hetero-knockout mouse brains were almost half of that in the wild littermates (data not shown). Body weight, locomotor activities, and performance in the rotarod test before and after 48 days of administration of HDL and reserpine were not significantly different between *Hspg2* hetero-knockout and wild litters (data not shown). There was a significant effect of genotype ( $F(1, 545) = 36.8$ ,  $p < 0.0001$ ), post-treatment time ( $F(4, 495) = 6.15$ ,  $p < 0.0001$ ), and treatment ( $F(3, 543) = 5.7$ ,  $p = 0.0008$ ) for the number of VCMs for 5 min. *Post hoc* analysis showed that the number of VCMs were significantly lower

in hetero-knockout mice than in wild-type mice after the last injection of HDL and reserpine after 48 or 49 consecutive days of administration of HDL and reserpine, and subsequent injection of physostigmine on the 50th day, or saline on the 53rd day (Figure 3b). The response of VCMs to physostigmine was subsequently evaluated (Figure 3c). There was a significant effect of genotype ( $F(1, 128) = 36.9$ ,  $p < 0.0001$ ), but not post-treatment time ( $F(4, 125) = 1.03$ ,  $p = 0.39$ ) for individual differences in the number of VCMs between pre-injection and post-treatment time. As for saline treatment, there was no significant effect of genotype ( $F(1, 118) = 0.13$ ,  $p = 0.72$ ) and post-treatment time ( $F(4, 115) = 0.31$ ,  $p = 0.87$ ). The numbers of VCMs were significantly reduced by injection of physostigmine compared with those before the injection at 24 h after HDL and reserpine injection in the wild-type mice but the differences in the numbers of VCMs before and after injection of physostigmine were not significant in hetero-knockout mice. The number of VCMs did not significantly alter after injection of saline in hetero-knockout mice and wild-type mice.

#### DISCUSSION

From a genome-wide association analysis, this study identified the role of *HSPG2* in neuroleptic-induced TD. The association was not significant in the initial screening and second confirmation after correction for multiple testing. However, screening with the tag SNPs for *HSPG2*,



**Figure 3** Analysis of vacuous chewing movements (VCMs) induced by haloperidol–reserpine in *Hspg2* knockout mice. (a) Schematic presentation of schedule of injections and measurements. (b) The average number (SEM) of VCMs for 5 min after injection. The abscissa axis shows the time after injection of HDL and reserpine, physostigmine, or saline. Significant difference between *Hspg2* hetero-knockout and wild-type mice is shown as \* at  $p < 0.05$ , \*\* at  $p < 0.01$ , and \*\*\* at  $p < 0.001$ . (c) Reduction of the number of VCMs after physostigmine or saline injection. Individual differences of the number of VCMs before injection (50th day for physostigmine or 53rd day for saline) to each time after injection grouped by the genotype are shown. Significant difference from mean = 0 is shown as \* at  $p < 0.001$ .

where the SNP (rs2445142) with the smallest  $p$ -value for association with TD in our genome-wide association study was located, identified one SNP (rs2124368) associated with TD even after correction for multiple testing. These two SNPs, which were found to be associated with TD, one identified by a genome-wide screening and another identified by screening with the tag SNPs, were not in LD. However, it is not obvious whether the finding for genetic association with TD of these SNPs in the *HSPG2* gene can be interpreted as significant, because of two steps of genome-wide association analyses before the step of screening of tag SNPs. Furthermore, the Human-1 BeadChip used in our initial screening is far from a complete genome coverage.

This may affect the credibility of the results. Confirmation of associations in other populations is necessary.

The SNP rs2445142 that showed the lowest association  $p$ -value in this study was associated with the expression levels of *HSPG2* in the human postmortem prefrontal cortex. The risk allele was associated with increased expression of *HSPG2*. The SNP rs2445142 is located in intron 1 of the *HSPG2* gene and is in complete LD with at least six SNPs located from introns 1–9. Among the SNPs associated with TD found in this study, the program TFSEARCH (<http://www.cbrc.jp/research/db/TFSEARCH.html>) predicts alteration of the transcription factor, LYF-1, binding affinity between the T and C alleles of rs897474 in intron 3.