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Association between major Multidrug Resistance 1 (MDR1) gene polymorphisms and plasma concentration of prolactin during risperidone treatment in schizophrenic patients

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Abstract

An in vitro study has suggested that risperidone is a substrate of P-glycoprotein, which is coded by *MDR1* gene. The rate of P-glycoprotein efflux transport can mediate brain penetration of lipophilic drugs. We therefore studied the effects of major polymorphisms of *MDR1* gene on plasma concentrations of prolactin. Subjects included *175* schizophrenic patients (68 males, *107* females) who were receiving 3 mg of risperidone twice daily for at least 4 weeks. Sample collections were conducted 12 h after the bedtime dosing. The plasma concentrations of prolactin in females were significantly higher than in males (54.3±27.2 versus 126.8±70.2 ng/ml, $p<0.001$). There was no difference in mean (±SD) plasma concentration of prolactin between *C3435T* genotypes [*C/C, C/T, T/T*; 62.3±33.3, 49.4±15.6, 53.2±33.2 ng/ml, ns] or *G2677T/A* genotypes [*G/G, G/T* or *A, T* or *A/T* or *A*; 58.0±27.7, 58.5±35.0, 46.1±20.7 ng/ml, ns] in males nor between *C3435T* genotypes (123.6±65.0, 127.8±79.2, 130.4±49.7 ng/ml, ns) or *G2677T/A* genotypes (123.3±67.0, 97.7±71.2, 144.9±69.9 ng/ml, ns) in females. Multiple regression analyses including plasma drug concentration and age revealed that plasma concentration of prolactin correlated with gender (standardized beta=0.540, $p<0.001$) and negatively with age (standardized beta=−0.183, $p<0.01$). No correlations were found between prolactin concentration and *MDR1* genotypes. These findings suggest that prolactin concentrations in females are much higher than in males but the major *MDR1* variants are not associated with the plasma concentration of prolactin.
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Keywords: Genotype; *MDR1*; Prolactin; Risperidone

1. Introduction

Hyperprolactinemia has been assumed to be an inevitable consequence of treatment with conventional antipsychotic agents (Petty, 1999). Major effects of hyperprolactinemia in women are amenorrhea, galactorrhea, cessation of normal cyclic ovarian function, loss of libido, occasional hirsutism and long-term risk of osteoporosis (Rubin, 1987; Petty, 1999). The effects in men include impotence, loss of libido, and hypospermatogenesis (Rubin, 1987; Petty, 1999). On the other hand, the prolactin response to antipsychotic drugs has been regarded as an index of antidopaminergic activity of antipsychotic drugs (Rubin, 1987). This has been partially confirmed by a PET study indicating a close relationship between prolactin response and dopamine D_2 receptor occupancy (Nordström and Farde, 1998). The prolactin response to antipsychotic drugs should therefore be evaluated not only from a clinical but also a pharmacological point of view.

Risperidone has a potent serotonin $5HT_2$ and a milder dopamine D_2 antagonistic activity (Schotte et al., 1995). However, adverse effects associated with hyperprolactinemia (Dickson et al., 1995; Kim et al., 1999; Popli et al., 1998) have been reported. With the exception of a retrospective analysis (Kleinberg et al., 1999), it has been demonstrated that the

Abbreviations: MDR1, Multidrug resistance 1; LC-MS-MS, Liquid chromatography-mass spectrometry-mass spectrometry; CV, coefficient variation; ANOVA, analysis of variance.

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pronounced hyperprolactinemia (Shiwach and Carmody, 1998; Caracci and Ananthamoorthy, 1999; David et al., 2000; Lavalaye et al., 1999; Yasui-Furukori et al., 2002) is consistently induced by risperidone treatment compared with conventional antipsychotic agents. However, inter-individual variability in plasma concentration during risperidone treatments has been implied (Yasui-Furukori et al., 2002). In addition, among women, the risperidone *dose* did not correlate with adverse events, nor did the adverse events correlate with endpoint prolactin levels (Kleinberg et al., 1999).

P-glycoprotein, which is encoded by *MDR1* gene, is involved in the acquisition of multidrug resistance phenotypes not only in cancer cells but also in normal tissues such as brain, kidney, liver and intestine (Thiebaut et al., 1987). Its major physiologic role is to serve as a barrier to entry and as an efflux mechanism for xenobiotics and cellular metabolites (Cordon-Cardo et al., 1989). Not only may P-glycoprotein limit intestinal drug absorption to constrain oral drug bioavailability, but the rate of P-glycoprotein efflux transport can also mediate brain penetration of lipophilic drugs (Ambudkar et al., 1999; Benet et al., 1999).

Recently, an in vitro study has shown that quetiapine and risperidone have stronger affinity to P-glycoprotein than other atypical antipsychotic drugs, suggesting that quetiapine and risperidone are substrates of P-glycoprotein (Boulton et al., 2002). Moreover, our in vivo study showed that verapamil increased risperidone exposure, suggesting that risperidone as a substrate of P-glycoprotein is clinically relevant (Nakagami et al., 2005).

Hoffmeyer et al. (2000) suggested that a single-nucleotide polymorphism in exon 26 of the *MDR1* gene (*C3435T*) was associated with a lower level of intestinal MDR1 expression. Moreover it has been reported that another single-nucleotide polymorphism in exon 21 of the *MDR1* gene (*G2677T/A*) is also linked with a lower function of P-glycoprotein (Siegmund et al., 2002).

We hypothesized that inter-individual variability in plasma prolactin concentration can be explained by major MDR1 polymorphism to some extent. Therefore, the effect of the *MDR1* gene polymorphisms on the plasma concentration of prolactin was examined in schizophrenic patients.

2. Methods

2.1. Subjects

The subjects were *175* schizophrenic Japanese inpatients (68 males and *107* females) who fulfilled the criteria for schizophrenia according to the Diagnostic and Statistical Manual of Mental Disorders, fourth edition. Some of the patients participated in our previous studies on the relationship between steady-state plasma drug concentrations and *CYP2D6* or MDR1 genotype (Mihara et al., 2003; Yasui-Furukori et al., 2003, 2004). The mean ± SD (range) of age, body weight and duration of illness were 43.2 ± 18.9 (18–75) years, 58.6 ± 12.6 (37–105) kg and 162 ± 128 (4–448) months, respectively. The study was approved by the Ethics Committee of Hirosaki University Hospital, and written informed consent to participate in this study was obtained from the patients and their families.

2.2. Protocol

The subjects had received risperidone 3 mg twice a day (8 a.m. and 8 p.m.) for 4 to 79 weeks. The elimination half-lives of risperidone and 9-hydroxyrisperidone were reported to be 3 to 20 h and 20 to 29 h, respectively. Therefore, plasma concentrations of these compounds already reached steady state in all of the subjects before initiating the study. The drugs coadministered were flunitrazepam 1–6 mg/day in 105 cases, diazepam 2–30 mg/day in 25 cases, lorazepam 1–3 mg/day in 12 cases and alprazolam 0.8–2.4 mg/day in 25 cases, biperiden 4–6 mg/day in 69 cases, trihexyphenidyl 4–10 mg/day in 18 cases, sennoside 12–60 mg/day in 71 cases.

2.3. Assays

Plasma concentrations of risperidone and 9-hydroxyrisperidone were measured using liquid chromatography-mass spectrometry-mass spectrometry (LC-MS-MS) method. Extraction procedure was as follows: 200 μl of 0.1 M phosphate buffer (pH 7), 50 μl of internal standard solution (R068808: Jansen Research Foundation) and 100 μl of methanol were added to 200 μl of plasma sample. Thereafter, 400 μl of 0.1 M Borax was added. The mixture is vortexed and poured over an Extrelut NT 1 (Merck) column, which is eluted with 7 ml of ethyl acetate. The eluate was evaporated under a nitrogen stream at 65 C°, and was redissolved in 100 μl of methanol which is again evaporated under a nitrogen stream at 65 C°. The residues were redissolved in 200 μl of acetonitrile/0.01 M ammonium acetate (50/50, pH 9.0), and 5 μl were injected onto the LC-MS-MS system. The system consisted of API 3000 (Sciex) and a column (Hypersil BDS C18 100 × 4.6, 3 μm). The mobile phase was gradient ammonium acetate (0.01 M, pH 9.0)-acetonitrile. Among the fragment ions of the compounds, the mass-to-charge ratio (m/z) 207.0 for risperidone, m/z 191.0 for 9-hydroxyrisperidone, and m/z 201.0 for the internal standard, were selected for ion monitoring. The lower limit of detection was 0.1 ng/ml for risperidone and 9-hydroxyrisperidone, and the values of the intra-assay and inter-assay coefficient of variation were less than 5% at all the concentrations (0.1–100 ng/ml) of calibration curves for both compounds.

Plasma prolactin concentration was determined using enzyme immunoassay (IMX Prolactin Dainapack, Dainabot,

Table 1
Clinical profiles, plasma drug concentrations and plasma concentration of prolactin in C3435T genotypes

	CC	CT	TT
	(n=54)	(n=82)	(n=39)
Age	46.6±15.0	45.5±16.2	42.8±15.4
Gender (male/female)	19/35	27/55	22/17
Plasma drug concentration (ng/ml)			
Risperidone	6.2±7.2	8.4±14.9	12.0±20.9
Nine-hydroxyrisperidone	48.6±15.6	41.5±17.1	45.2±21.8
Active moiety	47.7±30.0	49.9±23.8	57.2±28.8
Prolactin concentration (ng/ml)			
Total	102.0±62.9	102.0±75.0	86.8±56.2
Male	62.3±33.3	49.4±15.6	53.2±33.2
Female	123.6±65.0	127.8±79.2	130.4±49.7

Table 2
Clinical profiles, plasma drug concentrations and plasma concentration of prolactin in G2677T/A genotypes

	GG	GT or GA	TT, TA or AA
	(n=34)	(n=79)	(n=62)
Age	42.5±16.2	50.4±15.2	46.0±14.4
Gender (male/female)	15/19	31/48	22/40
Plasma drug concentration (ng/ml)			
Risperidone	8.4±14.7	7.6±8.6	9.3±17.4
Nine-hydroxyrisperidone	40.5±20.5	42.6±20.6	46.6±23.4
Active moiety	48.9±28.0	46.3±20.3	55.8±33.2
Prolactin concentration (ng/ml)			
Total	97.7±63.5	80.5±60.6	109.8±74.5
Male	58.0±27.7	58.5±35.0	46.1±20.7
Female	123.3±67.0	97.7±71.2	144.9±69.9

Table 4
Partial correlation coefficients and multiple correlation coefficient in multiple regression analyses in total, male and female subjects between prolactin level and various factors

Factors	Total subjects	Male	Female
	(N=175)	(n=68)	(n=107)
Age	−0.183**	−0.248	−0.236*
Plasma drug concentration (ng/ml)			
Risperidone	−0.113	0.079	−0.180
Active moiety	−0.065	0.093	0.056
MDR1 polymorphism			
C3435T	−0.031	−0.127	0.011
G2677T/A	0.084	−0.120	0.122
Gender	0.540***		
Multiple correlation coefficient	0.562***	0.317	0.297

*p<0.05, **p<0.01, ***p<0.001.

Osaka, Japan). The lowest limit of detection was 0.6 ng/ml, and inter-assay CV were 3.7, 3.5 and 3.5% at the concentrations of 8, 20 and 40 ng/ml for prolactin, respectively.

2.4. Analyses for MDR1 genotypes

For the determination of MDR-1 genotype, DNA was isolated from peripheral leukocytes by a guanidium isothiocyanate method. The C3435T alleles were detected by Taq-Man allelic discrimination methods (Verstuyft et al., 2003). G2677T/A alleles were identified using direct sequence methods (Horinouchi et al., 2002).

2.5. Data analyses and statistics

The comparison of several factors including the plasma concentration of prolactin and each MDR1 genotype was performed with use of one-way ANOVA. Multiple regression analyses were used to detect correlation between plasma concentration of prolactin and several factors including MDR1 genotypes (C3435T and G2677T/A), plasma drug concentration and age. The number of mutated alleles was used as independent variables and gender difference was analyzed using dummy variables (male=0, female=1). Because C3435T (Hoffmeyer et al., 2000) and G2677T/A (Siegmund et al., 2002) genotype proportionately expressed P-glycoprotein in the intestine, dummy variables were used for analyses of MDR1 genotype effects as follows: CC=2, CT=1 and TT=0 for C3435T, and GG=2, GA or TA=1 and TT, TA or AA=0 for G2677T/A. A p value less than 0.05 was regarded as statistically significant. All analyses were performed using SPSS 12.0J for windows (SPSS Japan Inc., Tokyo, Japan).

Table 3
Plasma prolactin concentration in combination of C3435T and G2677T/A

C3435T	G2677T/A	Male	Female
		(n=68)	(n=107)
C/C	G/G	71.0±36.3	104.4±34.2
C/C	G/T or A	43.6±15.1	128.9±90.5
C/C	T or A/T or A	46.9±12.9	147.7±74.9
C/T	G/G	56.2±25.0	136.5±82.7
C/T	G/T or A	48.6±10.3	75.1±45.3
C/T	T or A/T or A	54.1±18.8	147.9±79.3
T/T	G/G	73.6±24.5	112.3±33.1
T/T	T or A/T or A	53.5±17.4	137.9±54.7

3. Results

The patients had the following *MDR1* genotypes: *C/C* (54 cases), *C/T* (82) and *T/T* (39) for *C3435T* and *G/G* (34), *G/T* or *A* (79) and *T* or *A/T* or *A* (62) for *G2677T/A*, respectively. There was no difference in age or duration of illness between *C3435T* genotype or between *G2677T/A* genotype. However, plasma concentrations of risperidone and active moiety differed between C3435T, and plasma concentrations of 9-hydroxyrisperidone and active moiety differed between G2677T/A.

The plasma concentration of prolactin in females was significantly higher than males (137.4±81.6 ng/ml vs 56.8±30.9 ng/ml, p<0.001). Therefore, we analyzed prolactin concentration in males and in females separately. There were no differences in prolactin concentration in males or females between C3435T (Table 1) or between G2677T/A (Table 2). Plasma prolactin concentrations in the combination of these two genotypes (diplotypes) are shown in (Table 3). There was no difference in males (F=1.806, df=7, 60, p>0.05) or females (F=1.667, df=7, 98, p>0.05).

Multiple regression analyses including *C3435T* and *G2677T/A* allele for *MDR1*, gender difference and age showed that the plasma prolactin concentration correlated with gender (standardized beta=0.540, p<0.001) and negatively with age (standardized beta=−0.183, p<0.01), while there was no correlation between prolactin concentration and C3435T or G2677T/A (Table 4). When the multiple regression analyses without gender difference were further analyzed, the plasma prolactin concentration correlated negatively with age in females (standardized beta=−0.236, p<0.05), but not in males. Also, no correlations were found between prolactin concentration and MDR1 genotypes for C3435T (standardized beta=−0.031, ns) and G2677T/A (standardized beta=0.084, ns) (Table 4).

4. Discussion

P-glycoprotein is found in the epithelial cells lining the luminal surface of many organs often associated with an excretory or barrier function, i.e., the hepatic bile canalicular membrane, renal proximal tubule, villus-tip enterocyte in the small intestine, and the endothelial cells making up the blood–brain and blood–testes barriers (Ambudkar et al., 1999; Cordon-Cardo et al., 1989). A kinetic study showed large differences in brain concentration between the knockout animal, *mdr1a* (−/−) and *mdr1a/1b* (−/−) mice and normal animal, *mdr1a* (+/+) and *mdr1a/1b* (+/+) mice (Rao et al., 1999). Particularly, several animal studies showed that mdr1 knockout mice had extremely high brain concentrations of risperidone (Doran et al., 2005; Wang et al., 2004), suggesting that MDR1 protein plays an important role in penetration of risperidone from blood to brain. It is therefore more likely that *MDR1* variants affect risperidone concentration in brain, and hence prolactin concentration.

Contrary to our expectation, however, the plasma concentration of prolactin was not different between *MRD1* genotypes despite the fact that the active moiety concentration in the subjects with *MDR1* mutated alleles was higher than the subjects without *MDR1* mutated alleles. We have no clear explanation for this negative finding. A double-blind placebo-controlled in vivo study using healthy subjects demonstrated that prolactin response to a single oral dose of risperidone after verapamil treatment, which is a potent inhibitor of P-glycoprotein, was lower than placebo treatment (Nakagami et al., 2005). Therefore, it appears that MDR1 function in human blood–brain barrier and its role in prolactin concentration during risperidone treatment are unexpectedly complex.

Plasma concentrations of prolactin did not correlate with plasma drug concentration of risperidone or active moiety, although our previous studies showed positive correlation between plasma drug concentration and prolactin concentration during bromperidol and haloperidol treatments in males (Yasui et al., 1998; Yasui-Furukori et al., 2001). This discrepancy may be due to the relatively complex pharmacological profile of risperidone and its active metabolite, 9-hydroxyrisperidone. Risperidone has a potent serotonin $5HT_2$ and a milder dopamine D_2 antagonistic activity, (Schotte et al., 1995) and the $5HT_2$ antagonistic effect is associated with inhibition of prolactin secretion (Rubin, 1987).

It should be noted that the incidence of side effects related to hyperprolactinemia was not evaluated in the present study. In large clinical trials, risperidone-associated increase in serum prolactin levels did not correlate significantly with the emergence of possible prolactin-related side effects (Kleinberg et al., 1999). This may be due to the considerable number of patients who are without adverse effects despite having a high prolactin level. Nevertheless, prolactin monitoring during risperidone treatment should be conducted especially in premenopausal women who potentially suffer from potential adverse effects associated with hyperprolactinemia.

Patients receiving 6 mg/day of risperidone might have around 90% dopamine D2 receptor occupancy probably due to tight tight-binding to dopamine D2 receptor (Kapur and Seeman, 2001). According to the rapid dissociation model, tight-binding atypical agents are hypothesized to have an antipsychotic action when they cause other effects of dopamine blockade such as raised prolactin levels or extrapyramidal side effects (Kapur and Seeman, 2001). Although the MDR1 polymorphisms did not correlate with prolactin concentration, the possibility that the DRD2 variants rather than MDR1 may have predominant effect on dopamine D2 receptor occupancy of risperidone and other clinical responses cannot be excluded entirely.

An in vitro study has shown that quetiapine and risperidone have stronger affinity to P-glycoprotein than other atypical antipsychotic drugs, suggesting that quetiapine and risperidone are substrates of P-glycoprotein (Boulton et al., 2002). However, there is no in vivo data indicating that quetiapine or risperidone as a substrate of P-glycoprotein is of clinical relevance. Further information is required for antipsychotics to determine whether these *MDR1* genotypes are clinically relevant or not.

5. Conclusion

The present results indicate that the major *MDR1* polymorphisms are not associated with prolactin concentration during risperidone treatment in schizophrenic patients. Further studies will be valuable to determine whether or not *MDR1* genotypes are clinically relevant in the treatment with antipsychotics.

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Association Between Cytochrome P450 (CYP) 2C19 Polymorphisms and Harm Avoidance in Japanese

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Polymorphic enzyme cytochrome P450 (CYP) 2C19 is expressed not only in the liver but also in the brain and mediates the biotransformation of 5-hydroxytriptamine (5-HT). We investigated possible association between genetic polymorphism of CYP2C19 and individual personality traits, possibly influenced by neurotransmitters. Mentally and physically healthy Japanese subjects were enrolled in this study (n = 352). Temperament and Character Inventory (TCI) and *CYP2C19* genotyping were performed in all subjects. We detected CYP2C19*2 and *3 (http://www.imm.ki.se/CYPalleles/) using Amplichip CYP450 DNA tip. The number of genotypes classified as homozygous extensive metabolizer (EM), heterozygous EM, and poor metabolizer were 113, 181, and 58, respectively. Significant difference was found in TCI score in harm avoidance (HA; $F = 3.138$, $P < 0.05$). Post hoc analysis showed that TCI score in harm avoidance in homozygous EM was significantly lower than that in heterozygous EM ($P < 0.05$) or PM ($P < 0.05$). In sub-item analyses, HA3 (shyness with strangers, $P < 0.01$) and HA1 (anticipatory worry, $P < 0.05$) of TCI scores were significantly different among *CYP2C19* genotypes. Meanwhile, there were no differences in TCI scores of novelty seeking (NS; $F = 0.350$, n.s.), reward dependence (RD; $F = 1.080$, n.s.), or persistence (P; $F = 0.786$, n.s.) among *CYP2C19* genotypes. This study demonstrated that a significant association between CYP2C19 activity and HA is present in Japanese. © 2007 Wiley-Liss, Inc.

KEY WORDS: CYP2C19; harm-avoidance; personality; TCI

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INTRODUCTION

Brain cytochrome P450s (CYPs) were originally reported to occur at only 1% of the levels found in liver [Warner et al., 1988], but the levels of CYPs in specific neurons can be as high or higher than levels in hepatocytes [Miksys et al., 2002]. Although it is unlikely that brain CYPs contribute to overall clearance of xenobiotics, they are able to metabolize a variety of compounds, including many drugs that cross the blood–brain barrier to produce their pharmacological effects within the brain. Brain CYPs are also thought to participate in the metabolism of some neurotransmitters, endogenous steroids, and neurosteroids [Miksys and Tyndale, 2002]. Therefore, this aspect of their function may be important in influencing neural development and integration of overall brain function.

5-hydroxytryptamine (5-HT) is believed to be primarily metabolized by monoamine oxidase A, which deaminates 5-HT, yielding 5-hydroxyindole acetaldehyde that is converted to 5-hydroxyindole acetic acid (5-HIAA) by an aldehyde dehydrogenase. However, a recent in vitro study demonstrated that 5-HT relaxed precontracted isolated aortic rings, with or without endothelium in the presence of CYP2B6, 2C9, and 2C19, suggesting that 5-HT is biotransformed by CYP2B6, 2C9, and 2C19 [Fradette et al., 2004].

CYP2C19 enzyme is expressed not only in the liver but also in the brain [Miksys and Tyndale, 2002]. CYP2C19 mediates the metabolism of many drugs including antidepressants, benzodiazepine, and proton pump inhibitors [Desta et al., 2002]. The CYP2C19 polymorphism results in three phenotypic groups: homozygous extensive metabolizer (EM), heterozygous EM, and poor metabolizer (PM). Although more than 18 mutated alleles affecting CYP2C19 activity have been identified (see http://www.imm.ki.se/CYPalleles/), only CYP2C19*2 and *3 among these alleles are able to explain most phenotypes of CYP2C19 activity in Japanese.

Cloninger et al. [1993] demonstrated that human personality consists of seven dimensions including three temperament dimensions and four character dimensions, and on the basis of this model, Cloninger developed the Temperament and Character Inventory (TCI), a questionnaire for assessing personality traits. The four temperament dimensions, which include novelty seeking (NS), harm avoidance (HA), reward dependence (ED), and persistence (P), have been assumed to be related to monoamine neurotransmitters: NS with dopaminergic activities [Menza et al., 1993, 1995], HA with serotonergic activities [Demitrack et al., 1992; Stein et al., 1993], and RD with noradrenergic activities [Garvey et al., 1996; Curtin et al., 1997].

Therefore, the aim of the present study was to clarify the possible relationship between the genotypes of CYP2C19 polymorphism and behavioral traits in a large sample of Japanese, as measured by the TCI.

SUBJECTS AND METHOD

This study was carried out after obtaining approval from the Ethics Committee of Hirosaki University School of Medicine.

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The subjects enrolled consisted of all Japanese students in a medical school and medical staff (n = 352, M = 186, F = 166). Their mean (±SD) age was 29.9 ± 11.3 years (range: 18–69 years). After giving the subjects a full description of the study, written informed consent to participate was obtained from each of them. The subjects were asked to complete the 240 items of the Japanese version of the TCI, whose reliability and validity had been established by Kijima et al. [1996]. Genomic DNA was extracted from 5 ml of peripheral blood using DNA purification kit (QIAGEN). Genotypings of CYP2C19 were performed using AmpliChip CYP450 Test® DNA tip (Roche Diagnostics, Tokyo, Japan). This microarray-based assay screens for *CYP2C19*2* and *CYP2C19*3*, as well as 31 known mutations in *CYP2D6*. The subjects were allocated into three groups based on the number of mutated alleles for CYP2C19: homozygous EM (**1/*1*), heterozygous EM (**1/*2* or **1/*3*), and PM (**2/*2, *2/*3* or **3/*3*).

For statistical analysis, the SPSS for Windows ver 13.0 (SPSS Japan, Inc., Tokyo, Japan) software package was used. Genotype deviation from the Hardy–Weinberg equilibrium was evaluated by chi-squared test. The mean scores for the seven factors (novelty seeking, harm avoidance, reward dependence, persistence, self-directedness, cooperativeness, self-transcendence) of the TCI were compared among the three genotype groups using ANOVA. Post hoc analysis was performed using Scheffe test. $P < 0.05$ was regarded as significant.

RESULTS

The frequencies of genotypes for **1/*1, *1/*2, *1/*3, *2/*2, *2/*3*, and **3/*3* were 0.32, 0.34, 0.17, 0.065, 0.082, and 0.017, respectively. The number of homozygous EM, heterozygous EM, and PM were 113, 181, and 58, respectively. The genotype distribution was not significantly different from that expected according to the Hardy–Weinberg equilibrium. There was no difference in age ($F = 1.105$, df = 2, 349, $P < 0.332$) or gender ($F = 1.125$, df = 2, 349, $P < 0.326$) among these *CYP2C19* genotypes.

Significant difference was found in TCI score in harm avoidance (HA; $F = 3.138$, df = 2, 349, $P < 0.016$) (Table I). Post hoc analysis showed that TCI score in harm avoidance in homozygous EM was significantly lower than that in heterozygous EM ($P < 0.047$) or PM ($P < 0.048$). In sub-item analyses, HA3 (shyness with strangers) ($F = 6.656$, df = 2, 349, $P < 0.001$) and HA1 (anticipatory worry) ($F = 3.940$, df = 2, 349, $P < 0.020$) had significant difference in CYP2C19 genotype, while *CYP2C19* genotypes differed neither scores of HA2 (fear of uncertain) ($F = 0.460$, df = 2, 349, $P < 0.631$) nor HA4 (fatigability) ($F = 1.530$, df = 2, 349, $P < 0.218$) (Table II). There were no differences in TCI scores of novelty seeking (NS; $F = 0.350$, df = 2, 349, $P < 0.705$), reward dependence (RD; $F = 1.080$, df = 2, 349, $P < 0.314$), or persistence (P; $F = 0.786$, df = 2, 349, $P < 0.415$) among *CYP2C19* genotypes (Table I). In addition, self-directedness ($F = 1.647$, df = 2, 349, $P < 0.208$), cooperativeness ($F = 0.547$, df = 2, 349, $P < 0.579$), self-transcendence ($F = 2.261$, df = 2, 349, $P < 0.106$) did not differ among the *CYP2C19* genotype groups (Table I).

DISCUSSION

The results of this study showed that scores of TCI of HA were significantly different among *CYP2C19* genotypes in Japanese. TCI score of HA in homozygous EM was significantly lower than that in heterozygous EM or PM in this study. This is the first study suggesting association between CYP2C19 activity and some personality characteristics. Since CYP2C19 mediates biotransformation of 5-HT, low activity of CYP2C19 might be linked with high concentration of 5-HT in the brain. It might therefore be reasonable to correlate the activity of CYP2C19 with HA because it has been suggested that HA is associated with serotonergic activity [Demitrack et al., 1992; Stein et al., 1993].

5-TH has been believed to be primarily metabolized by monoamine oxidase A, which deaminates 5-HT, yielding 5-hydroxyindole acetaldehyde that is converted to 5-hydroxyindole acetic acid (5-HIAA) by an aldehyde dehydrogenase. However, metyrapone and ketoconazole increases 5-HT concentration in the brain [Kennett et al., 1985; Murphy, 1997; Leret et al., 1998; Healy et al., 1999]. Phenytoin, a drug biotransformed by CYP2C9 and CYP2C19, increase 5-HT in the brain without enhancing its synthesis [Chadwick et al., 1978; Pratt et al., 1985], suggesting that phenytoin competitively inhibits 5-HT biotransformation. In addition, in the presence of CYP2B6, 2C9, and 2C19, 5-HT relaxed precontracted isolated aortic rings, with or without endothelium, an effect prevented by the addition of methylene blue and an inhibitor of catalase, but not by myoglobin [Fradette et al., 2004]. In the absence of catalase, hydroxylamine was always assayed as a byproduct of 5-HT metabolism. CYP2B6, 2C9, and 2C19 biotransform 5-HT, yielding hydroxylamine, which is converted to nitric oxide in the presence of catalase.

Association between personality and another polymorphic enzyme CYP2D6 has been investigated. Bertilsson et al. [1989] investigated relationship between debrisoquine hydroxylation phenotype and personality in healthy Swedes using the KSP psychoasthenia scale. This study showed that PM for CYP2D6 had lower scores in KSP and a lack of hesitation in comparison to EM. Meanwhile, the second study reported that PM were more prone to anxiety and less successfully socialized than EM using the subjects of Spanish students [LLerena et al., 1993].

TABLE I. Distribution of the Temperament and Character Inventory (TCI) Subitem Scores Among the Number of Mutated Alleles for CYP2C19

	Number of mutated alleles			
	0 (n = 113)	1 (n = 181)	2 (n = 58)	Significance
Novelty seeking	21.3 ± 4.0	21.6 ± 5.2	21.1 ± 4.5	n.s.
Harm avoidance	18.7 ± 5.8	20.5 ± 6.1**	21.1 ± 6.7*	$P < 0.05$
Reward dependence	15.2 ± 2.9	14.7 ± 3.5	14.6 ± 3.2	n.s.
Persistence	4.5 ± 1.7	4.2 ± 1.9	4.4 ± 2.0	n.s.
Self-directedness	27.3 ± 6.7	26.0 ± 6.5	26.0 ± 6.8	n.s.
Cooperativeness	28.4 ± 5.3	27.8 ± 5.1	28.5 ± 5.5	n.s.
Self-transcendence	11.3 ± 5.6	10.0 ± 4.5	10.5 ± 4.8	n.s.

Data are mean ± SD.
*$P < 0.05$.
**$P < 0.01$, compared with subjects without mutated allele.

TABLE II. Distribution of the Harm Avoidance (HA) Subitem Scores Among the Number of Mutated Alleles for CYP2C19

	Number of mutated alleles			
	0 (n = 113)	1 (n = 181)	2 (n = 58)	Significance
Total HA	18.7 ± 5.8	20.5 ± 6.1**	21.1 ± 6.7*	$P < 0.016$
HA1 (anticipatory worry)	5.2 ± 2.3	5.8 ± 2.4	6.3 ± 2.8*	$P < 0.020$
HA2 (fear of uncertain)	5.3 ± 1.5	5.5 ± 1.7	5.4 ± 1.6	$P < 0.631$
HA3 (shyness with strangers)	4.3 ± 1.9	5.0 ± 1.7**	5.1 ± 2.0*	$P < 0.001$
HA4 (fatigability)	3.8 ± 2.2	4.1 ± 2.1	4.4 ± 2.4	$P < 0.218$

Data are mean ± SD.
*$P < 0.05$.
**$P < 0.01$, compared with subjects without mutated allele.

The discrepant results between the two studies might be explained by differences in the method for measuring debrisoquine hydroxylation capacity, the age of subjects, and the national character or race between Sweden and Spain. Another study in New Zealand showed that PM for CYP2D6 tends to be more novelty seeking and less harm avoidance than EM, using TCI. They used subjects that were recruited from long-term depressed patients [Roberts et al., 2004]. Although their findings were in accordance with Swedish findings but contrary to Spanish findings, this result should be carefully interpreted because depressed patients have lower scores of harm avoidance than controls.

The association between temperament and genetic variability within the serotonergic and dopaminergic neurotransmitter system has been investigated. For example a 44-bp polymorphism of the serotonin transporter gene (*5-HTT*) implies the relationship with harm avoidance [Lesch et al., 1996]. It is also reported that polymorphism of 48 bp repeat in dopamine D4 receptor gene (*DRD4*) has been associated with the novelty seeking category [Benjamin et al., 1996]. However, some studies have not been able to demonstrate an association between personality and polymorphisms [Ebstein et al., 1997; Blairy et al., 2000; Gebhardt et al., 2000; Kusumi et al., 2002], thus additional investigation that includes genetic polymorphisms, *CYP2D6*, *5-HTT*, and *DRD4* may be necessary to clarify the relationship between temperament and genetic polymorphisms within the serotonergic and dopaminergic neurotransmitter system.

Several studies have suggested that high HA is associated with depression [Farmer et al., 2003; Cloninger et al., 2006]. On the basis of the present result, it is possible that inter-individual variation of CYP2C19 activity is related with predisposition to depression. Likewise, a recent study suggests an association between *CYP2C9* gene and susceptibility to major depressive disorder due to an alteration in endogenous metabolism [LLerena et al., 2003]. Further studies are required to clarify the effect of CYP2C19 gene on susceptibility to major depressive disorder.

Our results failed to find association between CYP2C19 polymorphism and characters such as self-directedness, cooperativeness, and self-transcendence. This finding may be reasonable because characters are regarded as a product of learning according to Cloninger's Model.

In conclusion, this study demonstrated a significant association between inferred CYP2C19 activity and HA in Japanese. This result may depend upon the relative implication of CYP2C19 in the biotransformation of 5-HT.

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The G196A polymorphism of the brain-derived neurotrophic factor gene and the antidepressant effect of milnacipran and fluvoxamine

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Abstract

Prediction of the response to different classes of antidepressants has been an important matter of concern in the field of psychopharmacology. The purpose of the present study was to investigate whether the G196A polymorphism of the brain-derived neurotrophic factor (BDNF) gene is associated with the antidepressant effect of milnacipran, a serotonin norepinephrine reuptake inhibitor, and fluvoxamine, a selective serotonin reuptake inhibitor. The subjects of our previous study of milnacipran ($n = 80$) and fluvoxamine ($n = 54$) were included in the present study. Severity of depression was assessed with the Montgomery Åsberg depression rating scale (MADRS). Assessments were carried out at baseline and at 1, 2, 4 and 6 weeks of treatment. Polymerase chain reaction was used to determine allelic variants. In all subjects receiving milnacipran or fluvoxamine, the *G/A* genotype of the BDNF G196A polymorphism was associated with a significantly better therapeutic effect in the MADRS scores during this study. When milnacipran and fluvoxamine-treated subjects were analysed independently, the *G/A* genotype group showed greater reduction of MADRS scores than other genotype groups, irrespective of which antidepressant was administered. These results suggest that the BDNF G196A polymorphism in part determines the antidepressant effect of both milnacipran and fluvoxamine.

Keywords
antidepressant effect, genetic polymorphism, fluvoxamine, major depressive disorder, milnacipran

Introduction

Prediction of the response to different classes of antidepressants has been an important matter of concern in the field of psychopharmacology. A consistent relationship between the antidepressant effect and the plasma concentrations of selective serotonin (5-HT) reuptake inhibitors (SSRIs) has not been obtained (Burke and Preskorn, 1999), although early pharmacokinetic studies identified significant relationships between the antidepressant effect and plasma concentrations of several tricyclic antidepressants (Perry *et al.*, 1987). In terms of serotonin norepinephrine (NE) reuptake inhibitors (SNRIs), venlafaxine showed a positive association between antidepressant efficacy and plasma concentrations (Charlier *et al.*, 2002), while this relationship was not observed for milnacipran (Higuchi *et al.*, 2003).

Recent progress in pharmacogenetics has facilitated investigation of the relationship between genetic polymorphisms and the antidepressant response. Genetic polymorphisms of the 5-HT and NE transporter have been investigated intensively, because they are believed to be the primary target of SSRIs and SNRIs. As a result,

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several interesting findings have been reported (Malhotra *et al.*, 2004; Yoshida *et al.*, 2004), but there is no consistent evidence to predict the antidepressant response. Thus, further pharmacogenetic studies of antidepressants must be performed in order to predict the antidepressant response adequately.

Recently, it has been proposed that antidepressants eventually cause critical genes to be activated or inactivated, no matter how they act on receptors and enzymes (Stahl, 2000). One of the likeliest candidate genes is brain-derived neurotrophic factor (BDNF), which belongs to a family of neurotrophic factors including neurotrophin-3/4/5 and nerve growth factor and has an important role as a potent modulator of synaptic transmission and plasticity. Substantial evidence supports that BDNF is involved not only in cognitive processes, such as memory and learning, but also in the pathophysiology of mood disorders and in the mechanism of antidepressant action, as follows. Expression of BDNF mRNA is down-regulated by either acute or repeated stressful conditions of immobilization (Smith *et al.*, 1995). An antidepressant effect in both the learned helplessness and the forced swimming tests is observed as early as 3 days after a single infusion of BDNF into the hippocampus (Shirayama *et al.*, 2002). Chronic treatment with tranylcypromine, a monoamine oxidase inhibitor, caused a significant increase in BDNF mRNA in the rat hippocampus (Russo-Neustadt *et al.*, 1999), and chronic administration of amitriptyline, a tricyclic antidepressant, significantly increased BDNF protein levels in the rat hippocampus and prefrontal cortex (Okamoto *et al.*, 2003). Thus, the BDNF gene is a plausible candidate gene for mood disorders and pharmacogenetic studies of the antidepressant response.

The G196A polymorphism in exon IIIA is located within the propeptide region of the BDNF gene. Several association studies have examined the G196A polymorphism and vulnerability for bipolar or major depressive disorders (Hong *et al.*, 2003; Nakata *et al.*, 2003). These studies have found no major role for the polymorphism in the pathophysiology of mood disorders, although Egan *et al.* (2003) reported that it influences human memory and hippocampal function. So far only one pharmacogenetic study of antidepressants and the BDNF G196A polymorphism has been carried out (Tsai *et al.*, 2003); in this study, the response to treatment with fluoxetine was evaluated for only 4 weeks and the response rate was as low as 33.6%.

In the current 6-week study, we examined the effect of the BDNF G196A polymorphism on the antidepressant effect of milnacipran, an SNRI, and fluvoxamine, an SSRI. In addition, we investigated another polymorphism of C132T in the non-coding region of exon V of the BDNF gene, which was detected and named C270T by Kunugi *et al.* (2001). Plasma concentrations of milnacipran and fluvoxamine were investigated to evaluate patients' compliance and an influence on the antidepressant effect.

Materials and methods

Subjects

The subjects in our previous studies (Yoshida *et al.*, 2002; Yoshida *et al.*, 2004) were included in the present study. The subjects were Japanese patients who fulfilled DSM-IV criteria for a diagnosis of major depressive disorder and whose scores on the Montgomery Åsberg depression rating scale (MADRS) (Montgomery and Asberg, 1979) were 21 or higher. Patients with other axis I disorders (including dementia, substance abuse, dysthymia, panic disorder, obsessive–compulsive disorder and generalized anxiety disorder) and those with axis II disorders determined by clinical interview were excluded. Patients with a history of childhood disorders were also excluded, as were patients with severe non-psychiatric medical disorders. The patients were 20–69 years of age and had been free of psychotropic drugs at least 14 days before entry into the study. After complete description of the study to the subjects, written informed consent was obtained. This study was approved by the Ethical Committee of Akita University School of Medicine. The clinical characteristics of the patients are shown in Table 1. There was no significant difference between responders and non-responders in regard to sex, age, number of previous episodes and presence of melancholia. There was no significant difference in clinical characteristics when milnacipran and fluvoxamine-treated patients were analysed independently (data not shown). The number of previous depressive episodes was very low. Indeed, most of the patients (milnacipran: 64/80, fluvoxamine: 41/54) were in their first episode.

Milnacipran treatment

Milnacipran was administered twice daily (the same dose after dinner and at bedtime) for 6 weeks. The initial total daily dose was 50 mg/day, and after a week it was increased to 100 mg/day. Patients with insomnia were prescribed brotizolam, 0.25 or 0.5 mg, a benzodiazepine sedative hypnotic, at bedtime. No other

Table 1 Clinical characteristics of the patients in the milnacipran and fluvoxamine treatment (responders and non-responders)

	Responders ($n = 85$)	Nonresponders ($n = 49$)		p
Sex (male/female)	34/51	16/33	$\chi^2 = 0.72$	0.40[a]
Age (years) (± SD)	50.7 ± 12.4	52.2 ± 12.8	$t = -0.68$	0.50[b]
Number of previous episodes (± SD)	0.48 ± 1.7	0.33 ± 0.7	$t = 0.77$	0.44[b]
Melancholia (+/-)	21/64	15/34	$\chi^2 = 0.55$	0.46[a]

[a] Analysis performed with the use of the χ^2 test.

[b] Analysis performed with the use of the unpaired t test.

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psychotropic drugs were permitted during the study. Of 96 enrolled patients, ten did not complete the study; five patients because of side effects, one patient because of severe insomnia and four patients without explanation. Of the 86 patients who completed the 6-week study, six patients were excluded from the current analysis because plasma samples revealed very low milnacipran concentrations, indicative of poor compliance. Patients who completed the study included 52 women and 28 men, 49 outpatients and 31 inpatients, and ranged from 25 to 69 years of age (mean age = 51.4 ± 12.2 (±SD)).

Fluvoxamine treatment

Fluvoxamine was administered twice daily (the same dose after dinner and at bedtime) for 6 weeks. The initial total daily dose was 50 mg/day. The daily dose was increased to 100 mg/day after a week and was increased to 200 mg/day after another week. Concomitant administration of psychotropic drugs was restricted as in the milnacipran study. Of 66 enrolled patients, nine did not complete the study; four patients because of side effects and five patients without explanation. Of the 57 patients who completed the 6-week study, three patients were excluded from the current analysis because plasma samples revealed very low fluvoxamine concentrations, indicative of poor compliance. Patients who completed the study included 32 women and 22 men, 43 outpatients and 11 inpatients, and ranged from 24 to 69 years of age (mean age = 51.2 ± 13.2 (±SD)).

Data collection

Depression symptom severity was assessed with the use of the MADRS. Assessments were conducted at baseline and at 1, 2, 4 and 6 weeks after initiation of antidepressant treatment. A clinical response was defined as a 50% or greater decrease in the baseline MADRS score. Clinical remission was defined as a final MADRS score less than ten (Hawley *et al.*, 2002). Collection of blood samples was performed 12 hours after drug administration at bedtime, 4 weeks after initiation of each antidepressant treatment.

Genotyping

The BDNF G196A polymorphism was determined by a minor modification of the method of Tsai *et al.* (2003). The BDNF C132T polymorphism was determined by a minor modification of the method of Szekeres *et al.* (2003). Primers and enzymes used in this study were the same as previous studies; the conditions of the polymerase chain reaction and the chemical reagents were adjusted to our instruments.

Quantification of plasma milnacipran/fluvoxamine concentration

Plasma concentrations of milnacipran were measured with high performance liquid chromatography (HPLC). Details of the method have been described previously (Higuchi *et al.*, 2003). Plasma concentrations of fluvoxamine were measured with HPLC. Details of the method have been described previously (Ohkubo *et al.*, 2003). Genotyping and measurement of plasma concentrations were performed by laboratory personnel blind to the identity and clinical antidepressant effect of the patients. Moreover, clinicians were unaware of the genotyping results and the plasma milnacipran concentrations of each patient.

Statistical analysis

Differences in patient characteristics were analysed with the use of the unpaired t-test or chi-square test where appropriate. Differences in the MADRS scores during this study were examined with the use of two-way repeated measures analysis of variance (ANOVA), with genotype and time as factors. When significant interaction between factors was observed, contrasts were used to enable comparisons between each two of the three genotype groups. Differences in the MADRS scores at each evaluation point were examined with the one-way factorial ANOVA followed by the Fisher's PLSD test. Genotype deviation from Hardy-Weinberg equilibrium was evaluated by the chi-square test. Genotype distribution and allele frequencies were analysed with the use of the chi-square test. Plasma concentrations of milnacipran or fluvoxamine were analysed with the use of one-way factorial ANOVA in each genotype group; an unpaired t-test was then used to analyse differences between groups who were or were not responsive to milnacipran or fluvoxamine. Statistical analysis was performed using StatView version 5.0 (SAS Institute Inc., Cary, NC), except the two-way repeated measures ANOVA with contrasts was performed using SuperANOVA version 1.11 (Abacus Concepts, Inc., Berkeley, CA). Power analysis was performed with the use of G·Power (Buchner *et al.*, 1996). All tests were two-tailed; alpha was set at 0.05.

Results

Minor allele frequencies for the C132T polymorphism were very low and similar to those reported by Kunugi *et al.* (2001) and Szekeres *et al.* (2003); 5.0% in the patients treated with milnacipran and 3.7% in those treated with fluvoxamine. Therefore, only the G196A polymorphism was included in the statistical analysis. The observed genotype frequencies of the G196A polymorphism were within the distribution expected according to the Hardy-Weinberg equilibrium. As the authors reported that response to fluvoxamine was associated with allelic variations of the 5-hydroxytriptamine transporter gene-linked polymorphic region (5-HTTLPR) (Yoshida *et al.*, 2002) and response to milnacipran was associated with those of the norepinephrine transporter T-182C and G1287A polymorphisms (Yoshida *et al.*, 2004), it was necessary to confirm these polymorphisms to be controlled. The genotype distribution of these genotypes was not significantly different among the *G/G*, *G/A* and *A/A* genotype groups of the BDNF G196A polymorphism (data not shown).

Fig. 1 shows the MADRS scores over time in relation to the BDNF G196A polymorphism for all subjects receiving fluvoxamine or milnacipran. There was no significant difference in baseline MADRS scores among each genotype group. Two-way repeated

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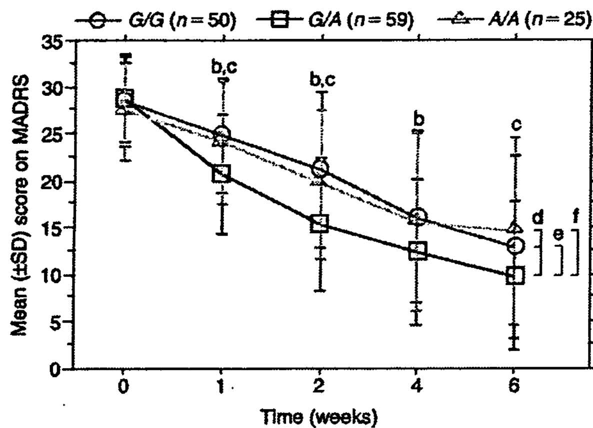

Figure 1 MADRS scores during 6 weeks of milnacipran/fluvoxamine treatment in three BDNF G196A genotype groups[a]

[a] Each point represents te mean score ± SD. Differences in the MADRS scores during this study were examined with the use of two-way repeated measures ANOVA with contrasts. Differences in the MADRS scores at each evaluation point were examined with the use of one-way factorial ANOVA followed by Fisher's PLSD test.
[b] Significant differences at each point between the *G/A* and *G/G* groups ($p = 0.0009$ at week 1, $p = 0.0001$ at week 2 and $p = 0.025$ at week 4).
[c] Significant difference at each point between the *G/A* and *A/A* groups ($p = 0.032$ at week 1, $p = 0.019$ at week 2 and 0.029 at week 6).
[d] Significant geneotype × time interaction among all three genotype groups ($F = 3.64$, $df = 8$, $p = 0.0004$).
[e] Signicant genotype × time interaction between the *G/A* and *G/G* groups ($F = 5.21$, $df = 4$, $p = 0.0004$)
[f] Significant genotype × time interaction between the *G/A/* and *A/A*: groups ($F = 3.99$, $df = 4$, $p = 0.0034$).

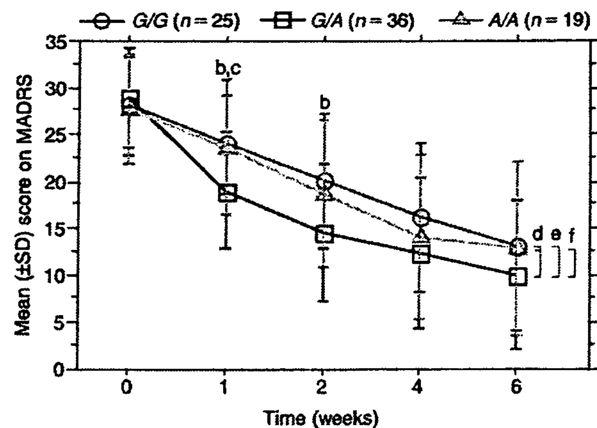

Figure 2 MADRS scores during 6 weeks of milnacipran treatment in three BDNF G196A genotype groups[a]

[a] Each point represents the mean score ± SD. Differences in the MADRS scores during this study were examined with the use of two-way repeated-measures ANOVA with contrasts. Differences in the MADRS scores at each evaluation point were examined with the use of one-way factorial ANOVA followed by Fisher's PLSD test.
[b] Significant difference at each point between the *G/A* and *G/G* groups ($p = 0.0031$ at week 1 and $p = 0.0056$ at week 2).
[c] Significant difference between the *G/A* and *A/A* groups ($p=0.011$ at week 1).
[d] Significant genotype × time interaction among all three genotype groups ($F = 2.30$, $df = 8$, $p = 0.021$).
[e] Significant genotype × time interaction between the *G/A* and *G/G* groups ($F = 3.54$, $df = 4$, $p = 0.0077$).
[f] Significant genotype x time interaction between the *G/A* and *A/A* groups ($F = 2.56$, $df = 4$, $p = 0.039$).

measures ANOVA including all three genotype groups indicated a significant genotype × time interaction. Contrast analysis indicated a significant genotype × time interaction between the *G/A* and *G/G* genotype groups. The MADRS score of the *G/A* genotype group was significantly lower than that of the G/G genotype group at 1, 2 and 4 weeks. Contrast analysis indicated a significant genotype x time interaction between the *G/A* and *A/A* groups. The MADRS score of the *G/A* genotype group was significantly lower than that of the *A/A* group at 1, 2 and 6 weeks. Contrast analysis indicated no significant genotype × time interaction between the *G/G* and *A/A* genotype groups ($F = 0.99$, $df = 4$, $p = 0.41$). There was no significant difference in the MADRS score at any evaluation point between the *G/G* and *A/A* genotype groups. When milnacipran- and fluvoxamine-treated subjects were analysed independently, the *G/A* genotype group showed greater reduction of MADRS scores than other genotype groups, irrespective of which antidepressant was administered (Figs. 2 and 3). Mean plasma concentrations of milnacipran were 92.3 ± 50.4 (±SD) ng/ml, 88.1 ± 31.1 ng/ml and 91.7 ± 36.2 ng/ml for the *G/G*, *G/A* and *A/A* genotype groups, respectively. There was no significant difference among the groups ($F = 0.99$, $df = 2, 77$, $p = 0.90$). Mean plasma concentrations of fluvoxamine were 169.1 ± 174.7 (±SD) ng/ml, 155.1 ± 118.6 ng/ml and 94.8 ± 35.3 ng/ml for the *G/G*, *G/A* and *A/A* genotype groups respectively. There was no significant difference among the groups ($F = 0.65$, $df = 2, 51$, $p = 0.53$).

Table 2 shows the genotype distribution and allele frequencies of responders and non-responders for all subjects receiving milnacipran or fluvoxamine. The proportion of responders was higher in *G/A* subjects than in subjects of other genotypes, but it did not reach a significant difference. There was no significant difference in the allele frequencies between responders and non-responders. The proportion of responders was non-significantly higher in *G/A* subjects than in subjects of other genotypes, irrespective of which antidepressants were administered (Table 3 and Table 4). When remitters and non-responders were compared, there was also no significant difference in the genotype distribution ($\chi^2 = 2.53$,

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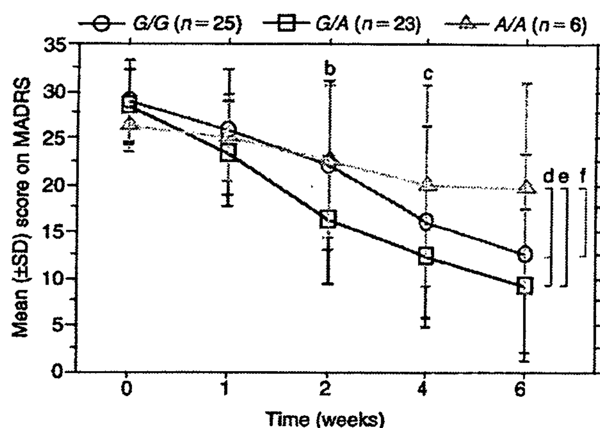

Figure 3 MADRS scores during 6 weeks of fluvoxamine treatment in three BDNF G196A genotype groups[a]

[a] Each point represents the mean score ± SD. Differences in the MADRS scores during this study were examined with the use of two-way repeated-measures ANOVA with contrasts. Differences in the MADRS scores at each evaluation point were examined with the use of one-way factorial ANOVA followed by Fisher's PLSD test.

[b] Significant difference between the *G/A* and *G/G* groups ($p = 0.015$ at week 2).

[c] Significant difference between the *G/A* and *A/A* groups ($p = 0.024$ at week 6).

[d] Significant genotype × time interaction among all three genotype groups ($F = 2.83$, $df = 8$, $p = 0.0053$).

[e] Significant genotype × time interaction between the *G/A* and *A/A* groups ($F = 4.55$, $df = 4$, $p = 0.0015$).

[f] Significant genotype × time interaction between the *G/G* and *A/A* groups ($F = 2.77$, $df = 4$, $p = 0.029$).

$df = 2$, $p = 0.12$ for the milnacipran treatment, $\chi^2 = 3.25$, $df = 2$, $p = 0.20$ for the fluvoxamine treatment and $\chi^2 = 4.26$, $df = 2$, $p = 0.12$ for both treatments) and genotype frequencies ($\chi^2 = 2.53$, $df = 1$, $p = 0.52$ for the milnacipran treatment, $\chi^2 = 0.64$, $df = 1$, $p = 0.64$ for the fluvoxamine treatment and $\chi^2 = 4.26$, $df = 1$, $p = 0.63$ for both treatments) (data not shown).

The plasma concentrations of milnacipran or fluvoxamine were not significantly different between responders and nonresponders, as shown in our previous studies (Yoshida *et al.*, 2002; Yoshida *et al.*, 2004).

This study of both milnacipran and fluvoxamine had a power of 0.16 to detect a small effect, 0.88 to detect a medium effect and 0.99 to detect a large effect in the genotype distribution ($n = 134$). For the allele frequency analysis ($n = 268$), this study had a power of 0.37 to detect a small effect, 0.99 to detect a medium effect and 0.99 to detect a large effect. In the power analysis, effect size conventions were determined according to the method of Buchner *et al.* (1996) as follows: small effect size = 0.10, medium effect size = 0.30 and large effect size = 0.50 (alpha = 0.05).

Discussion

The present study revealed that the BDNF G196A polymorphism affected the efficacy of both milnacipran and fluvoxamine. The *G/A* genotype of this polymorphism was associated with a significantly better therapeutic effect in the MADRS scores during this study, although the difference in final therapeutic response was not significant between the *G/A* and other genotype groups.

The results of this study are not well explained by the findings by Egan *et al.* (2003). Their human study showed that the *A* allele was associated with poorer episodic memory, abnormal hippocampal activation as determined by functional magnetic resonance imaging (fMRI), and lower hippocampal n-acetyl aspartate levels as assayed by MRI spectroscopy. According to their expression study, high concentrations of KCl induced detectable release of *G*-BDNF, whereas the activity-dependent release of *A*-BDNF was severely reduced and sometimes not detectable. Thus, the presence of the *G* allele is related to appropriate hippocampal function, neuronal function and activity-dependent BDNF release. In consideration of these findings, it is difficult to interpret the present results.

However, several aspects should be considered before trying to interpret our study based on the findings by Egan *et al.* (2003). First, the behavioural and mood abnormalities associated with major depressive disorder appear to result from disturbances mainly in the temporolimbic-frontal-caudate network (Drevets, 1999; Czeh *et al.*, 2001), although several lines of research support the notion that the hippocampus is also an important

Table 2 Genotype distribution and allele frequencies in responders and non-responders (milnacipran/fluvoxamine treatment)[a]

	Genotype distribution[b]			Allele frequency[c]	
	G/G	*G/A*	*A/A*	*G*	*A*
Responder	29 (34.1%)	43 (50.6%)	13 (15.3%)	101 (59.4%)	69 (40.6%)
Non-responder	21 (42.9%)	16 (32.6%)	12 (24.5%)	58 (59.2%)	40 (40.8%)

[a] Analysis performed with the use of the χ^2 test.

[b] No significant difference between responders and nonresponders ($\chi^2 = 1.32$, $df = 2$, $p = 0.12$).

[c] No significant difference between responders and nonresponders ($\chi^2 = 0.001$, $df = 1$, $p = 0.97$).

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Table 3 Genotype distribution and allele frequencies in responders and non-responders (milnacipran treatment)[a]

	Genotype distribution[b]			Allele frequency[c]	
	G/G	*G/A*	*A/A*	*G*	*A*
Responder	13 (26.0%)	26 (52.0%)	11 (22.0%)	52 (52.0%)	48 (48.0%)
Non-responder	12 (40.0%)	10 (33.3%)	8 (26.7%)	34 (56.7%)	26 (43.3%)

[a] Analysis performed with the use of the χ^2 test.
[b] No significant difference between responders and non-responders ($\chi^2 = 2.80$, df = 2, $p = 0.25$).
[c] No significant difference between responders and non-responders ($\chi^2 = 0.030$, df = 1, $p = 0.57$).

Table 4 Genotype distribution and allele frequencies in responders and non-responders (fluvoxamine treatment)[a]

	Genotype distribution[b]			Allele frequency[c]	
	G/G	*G/A*	*A/A*	*G*	*A*
Responder	16 (45.7%)	17 (48.6%)	2 (5.7%)	49 (70.0%)	21 (30.0%)
Non-responder	9 (47.4%)	6 (31.6%)	4 (21.0%)	24 (63.2%)	14 (36.8%)

[a] Analysis performed with the use of the χ^2 test.
[b] No significant difference between responders and nonresponders ($\chi^2 = 3.45$, df = 2, $p = 0.18$).
[c] No significant difference between responders and nonresponders ($\chi^2 = 0.53$, df = 1, $p = 0.47$).

region in the pathophysiology of major depressive disorder (Campbell and Macqueen, 2004). Therefore, the functional effect of the BDNF G196A polymorphism on the temporolimbic-frontal-caudate network is necessary to understand the present results adequately. However, such information is extremely limited; to our knowledge, there have been no reports investigating this issue using functional brain imaging, such as fMRI or positron emission tomography.

Only one cognitive study (Foltynie *et al.*, 2005) investigated the effect of the BDNF G196A polymorphism on performance of planning ability in Parkinson's disease using the Tower of London (TOL) task, a test of working memory (Robbins, 1996). The TOL task is reported to increase relative regional cerebral blood flow in the dorsolateral prefrontal cortex, lateral premotor cortex, rostral anterior cingulate cortex and dorsal caudate nucleus (Dagher *et al.*, 1999). Foltynie *et al.* (2005) revealed that the *A* allele of the BDNF G196A polymorphism was associated with better performance at the TOL task. This result is inconsistent with the results by Egan *et al.* (2003), who reported that the presence of the *A* allele was associated with impaired function in the hippocampus. The exact mechanism underlying this discrepancy is unclear. The study by Foltynie *et al.* (2005) was performed in Parkinson's disease not in major depressive disorder. However, it is possible that the functional effects of the BDNF G196A polymorphism differ among areas of the brain in major depressive disorder, and this regional difference in the temporolimbic-frontal-caudate network and the hippocampus may contribute to the better antidepressant effect in patients with the *G/A* genotype.

Additionally, some other studies indicated that subjects heterozygous for the BDNF G196A polymorphism have significant differences in expression of dichotomous or quantitative phenotypes than those homozygous for either allele. Momose *et al.* (2002) reported that homozygosity of the BDNF G196A polymorphism was more frequent in patients with Parkinson's disease. This finding suggests that the *G/A* genotype is less susceptible to Parkinson's disease than other genotypes. Tsai *et al.* (2003) reported a trend to a higher percentage change of the total Hamilton Depression Rating score for heterozygote patients in comparison to homozygote patients after fluoxetine treatment for 4 weeks. Their results are consistent with those of the present study and suggest that the *G/A* genotype is related to a favourable antidepressant effect. Besides the possible regionally different effects of the BDNF G196A polymorphism on brain function, another possibility is that the polymorphism may be in linkage disequilibrium with an as yet unidentified functional polymorphism with a molecular heterotic effect (Comings and MacMurray, 2000).

One major limitation of this study is the relatively small number of subjects, especially in the fluvoxamine arm. A second limitation is the relatively small end point treatment differences. These limitations may increase the possibility of a false positive and make it difficult to conclude that the BDNF G196A polymorphism is the common genetic factor for prediction of the antidepressant effect of both milnacipran and fluvoxamine. Further studies with a larger number of subjects are needed not only to confirm the results of this study but also to investigate the interaction of many genes, including the BDNF gene, on the mechanisms of antidepressant action.

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Association Study between Vesicle-Associated Membrane Protein 2 Gene Polymorphisms and Fluvoxamine Response in Japanese Major Depressive Patients

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Key Words

Depressive disorder · Fluvoxamine response · Haplotype analysis · Vesicle-associated membrane protein 2

Abstract

Background: Vesicle-associated membrane protein 2 (VAMP2) is a key component of the synaptic vesicle docking/fusion machinery and its mRNA reportedly increases in the frontal cortex of rats following chronic antidepressant and electroconvulsive treatment. VAMP2 is therefore thought to be involved in the mechanism of action of antidepressants and may alter their efficacy. The purpose of this study was to investigate whether the *VAMP2* gene is associated with clinical responses to a specific antidepressant, fluvoxamine. ***Methods:*** A total of 106 patients with major depressive disorder were given fluvoxamine (50–200 mg/day) for 8 weeks and assessed for severity of depression using the Semi-Structured Interview Guide of the Hamilton Depressive Scale (SIGH-D; 17 items) at 0 and 8 weeks. We defined a clinical response as more than a 50% reduction in baseline SIGH-D within 8 weeks, and defined clinical remission as a SIGH-D score of less than 7 at 8 weeks. Genotyping was performed by PCR-RFLP. ***Results:*** Analysis of haplotype tagging single nucleotide polymorphisms as well as haplotype analysis did not reveal any significant associations. ***Conclusion:*** Our results suggest that the *VAMP2* gene is unlikely to play a major role in the efficacy of fluvoxamine.

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Introduction

The selective serotonin reuptake inhibitors (SSRIs) are one of the first line drugs for treatment of major depressive disorder. Unfortunately, approximately 2 weeks are required for the onset of clinical effect, and only 60% of major depressive patients show a complete response to antidepressant treatment [1]. It is difficult for clinicians to predict which patients will respond to which drug based on clinical or biological features, although genetic factors are believed to play a major role in the variety of responses to treatment [2]. Many pharmacogenetic studies of the SSRI response have been undertaken, most of which have con-

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Table 1. Clinical characteristics of the patients in both definition groups

	Total patients	Males	Females	Age, years	Baseline SIGH-D	Fluvoxamine dose at 8 weeks mg/day	Previous episodes
Overall	106	49	57	43.2 ± 16.0	20.1 ± 6.5	122.0 ± 40.9	1.37 ± 0.56
Clinical response group[1]							
Responders	59	30	29	45.3 ± 16.6	21.5 ± 6.5	116.8 ± 43.4	1.34 ± 0.50
Nonresponders	47	19	28	41.0 ± 15.1	18.2 ± 5.9	128.9 ± 36.8	1.41 ± 0.59
p value		0.285		0.216	0.008	0.167	0.584
Clinical remission group[2]							
Remission	47	24	23	43.6 ± 14.7	19.2 ± 5.6	107.5 ± 43.2	1.34 ± 0.52
Nonremission	59	25	34	43.0 ± 17.0	20.7 ± 7.0	133.3 ± 35.5	1.39 ± 0.70
p value		0.373		0.694	0.23	0.002	0.698

Values for age, baseline SIGH-D, fluvoxamine dose and previous episodes are expressed as mean ± SD.
[1] Clinical response was defined as a 50% or greater decrease in the baseline SIGH-D score.
[2] Clinical remission was defined as a final SIGH-D score of less than 7.

centrated on mutations in the genes coding for pathways in the serotonergic systems [3–6]. However, no consistent pattern of results has been established [7]. Recently, researchers conducting SSRI pharmacogenetic studies have shifted their emphasis to genes other than those involved in serotonin concentration or receptor function [8, 9].

Vesicle-associated membrane protein 2 (VAMP2), which is located on synaptic vesicle membranes, is a key component of the regulated secretory pathway at nerve terminals and, along with syntaxin 1 and SNAP-25, forms the soluble N-ethylmaleimide-sensitive fusion protein attachment protein receptor (SNARE) complex [10–13]. Messenger RNA of *VAMP2* was recently reported to increase in the frontal cortex of rats following chronic antidepressant and electroconvulsive treatment [14]. Therefore, neuron secretory behavior alteration due to *VAMP2* gene expression was hypothesized to play a role in the mechanism of action of antidepressants and possibly to alter the clinical response of treated patients. In the present study, we investigated the effects of *VAMP2* gene polymorphisms on antidepressant responses to fluvoxamine in Japanese patients.

Materials and Methods

Subjects and Treatments

This study included 106 Japanese patients (49 males and 57 females; mean age ± standard deviation, 43.2 ± 16.0) who fulfilled the DSM-IV criteria for the diagnosis of major depressive disorder and whose scores on the 17 items of the Semi-Structured Interview Guide of the Hamilton Depressive Scale (SIGH-D) [15] were 12 or higher, indicative of their being in at least moderate depression [16]. In addition to these patients, 96 healthy volunteers, including unrelated medical staff and medical students, were recruited for linkage disequilibrium (LD) mapping. Patients with other axis I disorders, including schizophrenia, dementia, substance abuse, panic disorder, obsessive-compulsive disorder and generalized anxiety disorder, and those with axis II disorders diagnosed by the DSM-IV criteria were excluded by clinical interviews. All patients were free of psychotropic drugs in the past at least 1 month before beginning the study. After explaining the study to the subjects, written informed consent was obtained from each one of them. This study was approved by the Ethics Committee of the Nagoya University Graduate School of Medicine and Fujita Health University.

Fluvoxamine was administered two or three times a day for 8 weeks. The initial total dose was 50–100 mg/day and was gradually increased depending on the patient's condition. Patients with insomnia and severe anxiety were prescribed benzodiazepine drugs appropriately, but no other psychotropic drugs were allowed during the study.

Data Collection

Severity of depression symptoms was assessed using the 17 SIGH-D items. Assessments were conducted at baseline and 8 weeks after initiating antidepressant treatment. A single evaluator performed all the ratings for a single patient. A clinical response was defined as a 50% or greater decrease in the baseline SIGH-D score, and clinical remission was defined as a final SIGH-D score of 7 or less. The clinical characteristics of the patients, classified according to these definitions, are shown in table 1.

Single Nucleotide Polymorphism Selection and Genotyping

Genomic DNA was extracted from the peripheral blood of all subjects. For LD mapping, we selected single nucleotide polymorphisms (SNPs; rs8067606, rs1061032, rs2278637) distributed equally throughout the gene based on data from dbSNP (http://www.ncbi.nlm.nih.gov/projects/SNP/; fig. 1). These SNPs were first genotyped in the 96 control samples to avoid redundant genotyping, and then LD blocks were determined with reasonable criteria based on 95% confidence intervals on the D′ values using Haploview v3.2 software (http://www.broad.mit.edu/mpg/haploview/). Next, haplotype-tagging SNPs (htSNPs) were selected within each LD block to provide 90% haplotype coverage. Finally, we genotyped each selected htSNP in the depressive samples using PCR-RFLP. Detailed protocols for the PCR-RFLP method are available upon request.

Statistical Analysis

Genotyping deviation from Hardy-Weinberg equilibrium was evaluated by a χ^2 test using the Haploview software. Marker-trait allele/genotype-wise association was evaluated using a conventional χ^2 test (SPSS version 11.0J, Tokyo, Japan) and haplotype-wise was evaluated using COCAPHASE software (http://www.rfcgr.mrc.ac.uk/~fdudbrid/software/unphased/). The COCAPHASE program performs log-likelihood ratio tests under a log-linear model for global p values. To estimate the haplotype frequencies of htSNPs, we used the expectation-maximization algorithm. Global p values were calculated for haplotype-wise analyses, and we also performed exploratory analysis of the possible correlations between response or remission, fluvoxamine treatment, and several clinical factors by logistic regression (SPSS). In these analyses, response classification was set as the dependent variable, and gender, age at the time of recruitment, fluvoxamine dose at 8 weeks, SIGH-D total score at the baseline, and htSNP genotypes were set as the independent variables. The significance level for all statistical tests was $p < 0.05$.

Results

After three SNPs were genotyped to evaluate the LD in the control samples, one LD block was defined and two SNPs (rs1061032 and rs8067606) were selected as the htSNPs for this gene (fig. 1). Genotyping these two htSNPs in all the depressive samples revealed that all the respective genotypic frequencies of these SNPs were in accordance with Hardy-Weinberg equilibrium.

In the allele/genotype-wise association analysis, the frequencies of each htSNP were not significantly different between fluvoxamine responders and nonresponders, or between remitters and nonremitters (table 2). In the haplotype-wise association analysis, we found no significant associations between fluvoxamine responders and nonresponders, or between remitters and nonremitters (table 3).

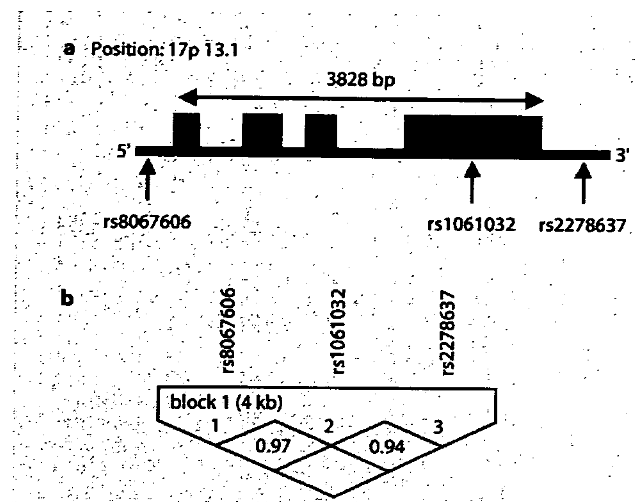

Fig. 1. Genomic structure and pairwise LD of *VAMP2*. **a** Genomic structure of VAMP2 and SNPs used in association analyses and LD mapping. The vertical bars represent exons of VAMP2, and the numbers under the arrows represent SNP IDs. **b** Pairwise LD of VAMP2 in the 96 controls. Each block was defined by a solid spine of the LD using Haploview v3.2. The numbers in the polygons represent D′, and the blank space represents complete LD.

In our exploratory logistic regression analysis, none of the htSNP genotypes correlated with clinical responses or remission (data not shown).

Discussion

In this study, the polymorphisms and estimated haplotypes of *VAMP2* were not associated with response or remission in fluvoxamine-treated Japanese depressive subjects. The exploratory logistic regression analysis revealed that none of the htSNP genotypes could serve as a predictor for clinical response or remission.

VAMP2 forms the SNARE complex at the presynapse, and SNARE proteins are central to the membrane fusion machinery. Membrane fusion is regulated by a Ca^{2+} sensor, and synaptotagmin is believed to function as a Ca^{2+} sensor, not only binding to the membrane in a Ca^{2+}-dependent manner but also interacting with SNARE complexes [17]. As VAMP2 interacts with various proteins that are essential for vesicular transport and/or fusion, other genes interacting with VAMP2 might play important roles in the response to antidepressants. We must therefore consider gene-gene interactions between *VAMP2* and these other genes [18].