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Gene expression and association analysis of vascular endothelial growth factor in major depressive disorder

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Abstract

Vascular endothelial growth factor (VEGF) has been implicated in neuronal survival, neuroprotection, regeneration, growth, differentiation, and axonal outgrowth, which are known to be involved in the pathophysiology of major depressive disorder (MDD). Recently, the VEGF mRNA expression in the peripheral leukocytes from Alzheimer's disease or cardiovascular disease was reported to be changed. We hypothesized that the expression of the VEGF mRNA in the peripheral leukocytes may be a good candidate for the biological marker for MDD. Thirty two patients with MDD and age- and sex-matched control subjects were included in this expression study. The VEGF mRNA levels in the peripheral leukocytes from drug-naive MDD patients were significantly higher than those from the control subjects and the magnitude of the decrease of VEGF mRNA after 8-week treatment significantly correlated with clinical improvement. Then, we genotyped two single nucleotide polymorphic markers of VEGF gene, which were reported to be associated with amyotrophic lateral sclerosis and Alzheimer's disease, in patients with MDD and control subjects ($n=154$, each). We did not find any significant association between these markers and MDD or its clinical subtypes. Our investigation indicates that the higher expression levels of VEGF mRNA in the peripheral leukocytes are associated with the depressive state and their recovery after treatment may be associated with the clinical improvement.
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Keywords: Gene association; Gene expression; Major depressive disorder; Paroxetine; VEGF

1. Introduction

Recent molecular and cellular studies of major depressive disorder (MDD) and antidepressants have moved the field of mood disorder research beyond the monoamine hypothesis of MDD. These studies demonstrate that stress and antidepressant treatment exert opposing actions on the expression of specific neurotrophic factors in limbic brain regions involved in the regulation of mood and cognition. Most notable are studies of brain-derived neurotrophic factor. However, more recent studies demonstrate that vascular endothelial growth factor (VEGF) increases the proliferation of neurons in the adult hippocampus and has been implicated in a vascular niche hypothesis of adult neurogenesis (Palmer et al., 2000). VEGF is an angiogenic cytokine able to induce vascular endothelial cell proliferation, migration, and vasopermeability in many type of tissues (Ferrara et al., 2003) and is produced by a number of cell

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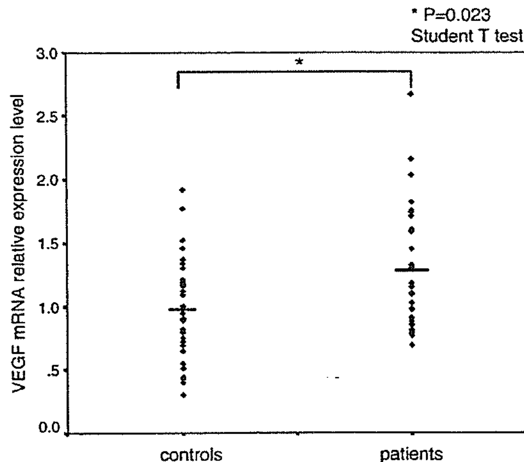

Fig. 1. VEGF mRNA expression levels before treatment. Bars indicate the mean of the values of each subject (control, age- and sex-matched controls, $N=32$; baseline, patients before treatment, $N=32$). The VEGF mRNA levels were significantly higher in patients (control; 0.92±0.21, baseline; 1.23±0.5: Student t test $P=0.023$).

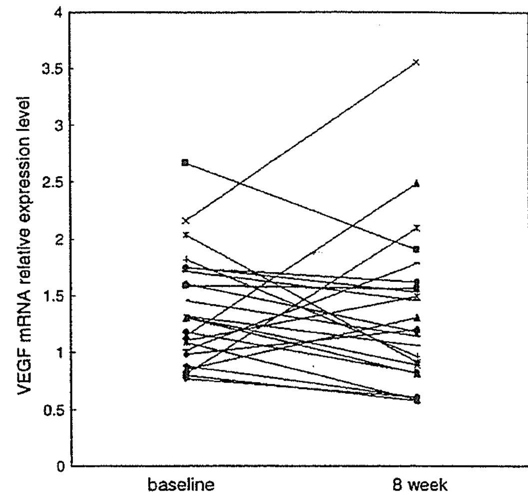

Fig. 2. VEGF mRNA expression levels during the treatment. Patients before treatment ($N=24$) and patients after 8-week paroxetine treatment ($N=24$) are connected by the solid lines. The VEGF mRNA levels did not show a significant change during the treatment (baseline; 1.37±0.49, 8-week; 1.34±0.69, paired t test $P=0.763$).

2.5. Genotyping

Genotyping of C-2578A (rs699947) and G-634C (rs2010963) was performed using commercially available TaqMan probes and Applied Biosystems 7500 Fast Real-time PCR System according to the protocol recommended by the manufacturer (Applied Biosystems, California, USA).

2.6. Statistical analysis

Statistical calculations were carried out using the SPSS Statistical Software Package 11.5 (SPSS, Tokyo, Japan). Expressional differences between patients and control subjects were calculated using the Student T test. Change before and after treatment was calculated with the paired T test. Pearson correlation coefficients were used to evaluate the correlations between VEGF mRNA levels and either paroxetine concentration or SIGH-D score. All significance levels were two-sided. The distribution of genotypes and alleles in the two study groups was compared using the Fisher's exact test. Haplotypic associations were examined with HAPLOVIEW software (Barrett et al., 2005). One-way ANOVA was performed to assess the possible association between the VEGF genotype and clinical subtypes, including sex, age of onset, psychotic feature, suicidal behavior and family history. The criterion for significance was set at $P<0.05$ for all tests. Data are presented as mean±standard deviation.

3. Results

3.1. VEGF mRNA expression in major depression and control subjects

The relative amount of VEGF mRNA in the peripheral leukocytes was standardized with GAPDH mRNA as an internal standard. There was no significant difference of VEGF mRNA expression between males (mean±S.D., 1.11±0.41) and females (mean±S.D., 1.10±0.50). There was no significant correlation between VEGF mRNA levels and age in either patients (Pearson correlation test: $P=0.326$) or controls (Pearson correlation test: $P=0.511$). There was no significant correlation between VEGF mRNA levels and leukocytes count at baseline (6.28±1.64) in patients (Pearson correlation test: $P=0.505$). VEGF mRNA levels was in the range of 0.30–1.92 (0.97±0.41) in 32 healthy volunteers, while 0.69 – 2.67 (1.23±0.5) in 32 medication free depressed patients, showing a statistical difference (Student T test: $P=0.023$; Fig. 1). VEGF mRNA levels at baseline was 1.33±0.53 in 23 first episode patients, while 0.99±0.29 in 9 recurrent episode patients, showing no statistical difference but a trend to be higher in first episode (Student T test: $P=0.077$). The mean SIGH-D scores at baseline (total:21.3±7.5) was 19.6±6.5 in 23 first episode patients, while 25.7±8.4 in 9 recurrent episode patients,

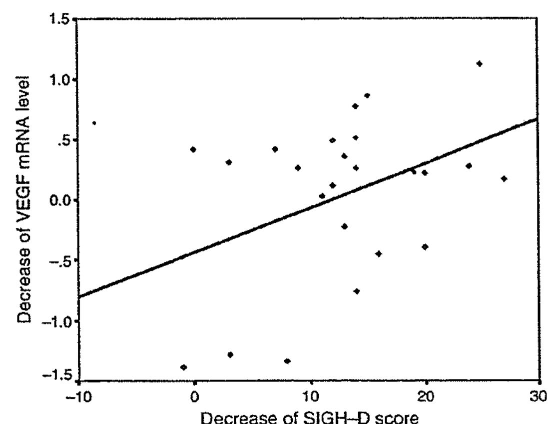

Fig. 3. Significant correlations between the decrease of VEGF mRNA and improvement of SIGH-D score (Pearson correlation $r=0.41$ $P=0.049$, $N=24$). Each point represents an individual and the solid lines represent the regression of the correlations.

types including macrophages, keratinocytes, T cells, and mesangial, kidney, and smooth muscle cells, as well as by various tumor cells (Berse et al., 1992; Brown et al., 1992; Freeman et al., 1995). VEGF has also been implicated in neuronal survival, neuroprotection, regeneration, growth, differentiation, and axonal outgrowth (Greenberg and Jin, 2004; Sun et al., 2003). Exposure to unpredictable stress decreases the expression of VEGF in hippocampus (Heine et al., 2005). Expressions of VEGF are increased after electroconvulsive seizure therapy (ECS) which is the most effective treatment for depression (Newton et al., 2003).

Recently, several studies including our own have shown altered mRNA expressions in the peripheral leukocytes of patients with mental disorders (Rocc et al., 2002; Iga et al., 2005, 2006). Since VEGF mRNA is expressed both in the brain and in the peripheral leukocytes and down regulation of VEGF production has been recently demonstrated by the peripheral immune cells of patients with Alzheimer's disease (Solerte et al., 2005), we hypothesized that the expression level of VEGF mRNA in the leukocytes would also be a candidate marker of major depressive disorder. Thus, we conducted a study for comparing the expression level of VEGF mRNA in the leukocytes of undedicated MDD patients with healthy controls. The expression levels were measured again after 8-week treatment with paroxetine.

The human VEGF gene is located on chromosome 6p21.3 and consists of eight exons (Tischer et al., 1991). A number of polymorphisms localized in the promoter region of VEGF gene are associated with several neuropsychiatric diseases such as amyotrophic lateral sclerosis, Alzheimer's disease and vascular dementia (Lambrechts et al., 2003; Del Bo et al., 2005; Kim et al., 2006). However, there is no evidence supporting a role of its genetic susceptibility to MDD. Thus, we conducted a genetic association analysis of VEGF gene polymorphisms.

2. Materials and methods

2.1. Subjects for expression analysis

The subjects consisted of 32 patients with major depressive disorder (10 males, 22 females and mean age 42.7±12.6) and 32 age- and sex-matched controls (10 males, 22 females and mean age 43.1±12.7). Before study participation, all subjects signed an informed consent form approved by the Ethical Committee of the University of Tokushima Graduate School. All patients were diagnosed as Major Depressive Disorder according to DSM-IV (APA 1994) by at least two trained psychiatrists. All subjects underwent extensive medical, neurological, psychological and laboratory evaluations before participating in the study. The persons who had axis II disorders were removed from the study. The diagnosis and the eligibility of the patients were reconfirmed during follow-up periods. Twenty three patients were in the first and other nine were in the recurrent depressive episode. All patients did not receive any antidepressants for the current episode before blood sampling. All patients were treated with paroxetine for 8 weeks. The dose of paroxetine was started with 10 or 20 mg for the first 2 weeks and gradually increased up to 40 mg based on judgment of the trained clinician. At baseline and 8-week, subjects were rated with Structured Interview Guide for the 17-item Hamilton Depression Rating Scale (SIGH-D 17, Williams JB 1988; Japanese version, Nakane Y 2000) before blood collection. Peripheral blood was also collected from 32 sex- and age-matched volunteers who were in good physical health with a history of neither psychiatric nor serious somatic diseases and were not taking any medication. Almost all samples are collected in the morning. Besides, we found no apparent fluctuation in the expression levels in blood samples from the same person taken at 9:00, 14:00 and 19:00 in a day. Probands who had first-degree relatives with psychiatric disorders were excluded from the control subjects.

2.2. Subjects for genetic analysis

Samples were collected at the Tokushima University hospital and the Ehime University Hospital. These included 154 patients with major depression (70 males, 84 females and mean age 45.1± 15.2) and 154 age–sex-matched control subjects (70 males, 84 females and mean age 45.0±14.0). Patients were diagnosed with the consensus of at least two trained psychiatrists using DSM-IV (APA 1994). Controls were selected by the same criteria as the expression study. All these subjects were Japanese, unrelated to each other, and living in the same area (Shikoku Island in Japan). All participants signed an informed consent form approved by the Ethical Committee of the University of Tokushima Graduate School or Ehime University.

2.3. Quantification of blood paroxetine concentration

The paroxetine quantification was performed using high performance liquid chromatography with 4-fluoro-7-nitrobenzo-2-oxa-1,3-diazole (NBDF)-derivatization, according to the method of Irie et al. (2000) with slight modification in that the separation was performed on a Phenomenex C18 column (4.6×250 mm).

2.4. Quantitative real-time PCR

Total RNA was extracted from the peripheral leukocytes of whole blood samples using the PAX gene Blood RNA kit (Qiagen, Tokyo, Japan) according to the manufacturer's recommendations. Residual genomic DNA was digested with RNase-free DNase I (Qiagen). 2 μg of total RNA was used for cDNA synthesis by random (N6) primers and Quantiscript Reverse Transcriptase (Qiagen, Tokyo, Japan) after assessing RNA quality and quantity with NanoDrop (NanoDrop Technologies, Delaware, USA). For quantitative PCR method, we used commercially available TaqMan probe according to the manufacturer's recommendations (Applied Biosystems, CA, USA). We used a control gene (Glyceraldehyde-3-phosphate dehydrogenase: GAPDH) for normalization of possible fluctuations in quantitative values of the target transcripts (Applied Biosystems). Measurements of each gene expression were conducted in triplicate.

Table 1
VEGF polymorphisms Genotype distributions and allele frequencies

Group		Genotypes, *n* (%)				Allele frequencies *n* (%)		
G-634C	HWE	G/G	G/C	C/C	P	G	C	P
Patients								
Total	0.13	51(33.1)	67(43.5)	36(23.4)	0.27	169(54.9)	139(45.1)	0.69
Single	0.05	32(42.1)	28(36.8)	16(21.1)	0.08	92(60.5)	60(39.5)	0.41
Recurrent	1.00	19(24.3)	39(50.0)	20(25.7)	0.30	77(49.4)	79(50.6)	0.15
Controls	0.48	47(30.5)	80(51.9)	27(17.5)		174(56.5)	134(43.5)	
C-2578A	HWE	C/C	C/A	A/A	P	C	A	P
Patients								
Total	0.21	79(51.3)	58(37.7)	17(11.0)	0.21	216(70.1)	92(29.9)	0.93
Single	0.06	36(36.8)	27(44.7)	13(18.5)	0.04	99(65.1)	53(34.9)	0.40
Recurrent	0.60	43(55.1)	31(39.7)	4(5.2)	0.42	117(75.0)	39(25.0)	0.23
Controls	0.20	71(46.1)	72(46.8)	11(7.1)		214(69.5)	94(30.5)	

showing no statistical difference but a trend to be higher in recurrent episode (Student *T* test: $P=0.062$). No significant relationships between baseline VEGF mRNA levels and baseline SIGH-D score (Pearson correlation test: $p=0.466$) or leukocytes count at baseline ($6.28\pm1.64\times1000/\mu l$) (Pearson correlation test: $P=0.505$) were observed.

3.2. VEGF mRNA expression after paroxetine treatment

We could follow up 24 patients during 8-week treatments. Mean paroxetine doses were 31.0±9.1 mg/day at 8-week treatments. Mean paroxetine concentration was 78.5±60.5 ng/ml at that point. Depressive symptoms were significantly improved after 8-week paroxetine treatments (SIGH-D scores at baseline and 8-week, 20.8±7.2 and 7.8±6.5, respectively; paired *T* test: $P<0.001$). The VEGF mRNA level in the leukocytes was not significantly changed at 8-week from baseline (1.37±0.49 at baseline; 1.34±0.69 at 8-week; paired *T* test: $P=0.763$; Fig. 2). There was no significant change of leukocytes count between baseline ($6.28\pm1.64\times1000/\mu l$) and 8-week ($6.26\pm1.45\times1000/\mu l$) (paired *T* test: $P=0.40$) and no correlation between VEGF mRNA levels and leukocytes count at 8-week (Pearson correlation test: $P=0.447$). The decrease of VEGF mRNA did not show a significant correlation with paroxetine concentrations (Pearson correlation test $P=0.495$) and changes of leukocytes count (Pearson correlation test $P=0.674$). However, the decrease of the VEGF mRNA levels show a significant correlation with the improvement of SIGH-D score (Pearson correlation $r=0.41$ $P=0.049$; Fig. 3).

3.3. Genetic association analysis

We genotyped two single nucleotide polymorphic (SNP) markers (C-2578A (rs699947) and G-634C (rs2010963)). There were no significant deviations in those two SNPs from the Hardy–Weinberg equilibrium in either patients or control subjects. The genotypic distribution of rs699947 of 0.30 in this study is consistent with that of HapMap project reporting 0.32 in Japanese population. Genotype and allele frequencies of the two SNPs are shown in Table 1. Haplotype analysis of the two SNPs is also shown in Table 2. Analysis of genotype effects on each clinical subtype (age, sex, age of onset, psychotic features, suicidal behavior and family history) is shown in Table 3. To control for type I error in gene association study given the number of statistical tests, we used a Bonferroni correction to test the null hypothesis that VEGF gene polymorphisms had no association with MDD (critical alpha=.05/12=.0042) and had no effect on the clinical subtypes (critical alpha=.05/5=.001). There were no significant associations between these SNPs and MDD. We also could not find any significant association of each genotype with clinical subtypes. Because our sample size had a enough post-hoc power of 0.99 to detect an effect size of $w=0.5$ (moderate) at the 0.05 significance level (two tailed) calculated by soft ware program G*Power (http://www.psycho.uni-duesseldorf.de/aap/projects/gpower) (Erdfelder et al., 1996), the failure to demonstrate a significant association may not reflect type II error due to the small sample size.

Table 2
VEGF polymorphisms haplotype distributions

C-2578A	G-634C	Frequency	Patient/control ratios	*P*
C	C	0.443	0.451/0.435	0.69
A	G	0.302	0.299/0.305	0.86
C	G	0.255	0.250/0.260	0.78

Table 3
Demographic data and clinical characteristics of the VEGF polymorphisms within the depressive patients

G-634C	G/G(n=51)	G/C(n=67)	C/C(n=36)	*F*	*P*
Sex	24/27	30/37	16/20	0.04	0.961
Age of onset	40.3(14.1)	39.7(12.9)	42.7(14.4)	0.55	0.576
Psychotic feature	6/45	6/61	5/31	0.31	0.737
Suicidal behavior	4/47	12/55	7/29	1.53	0.220
Family history	15/36	20/47	12/24	0.09	0.916
C-2578A	**C/C(n=79)**	**C/A(n=58)**	**A/A(n=17)**	***F***	***P***
Sex	34/45	28/30	8/9	0.19	0.826
Age of onset	41.9(14.5)	37.7(12.8)	44.4(10.7)	2.42	0.092
Psychotic feature	8/71	6/52	3/14	0.42	0.658
Suicidal behavior	14/65	7/51	2/15	0.49	0.614
Family history	27/52	16/42	4/13	0.56	0.575

(Age, Age of onset: Mean (SD), Sex: Male/Female, Psychotic feature, suicidal behavior, family history: with/without).

4. Discussion

The present study is the first report on gene expression in the peripheral leukocytes and gene association analysis of VEGF gene in MDD. There are three major findings in our investigation.

First, the mean VEGF mRNA levels in the peripheral leukocytes of depressive patients before treatment (baseline) were significantly higher than those of age- and sex-matched controls. Although VEGF is thought to be an important factor in pathophysiology of MDD because of its neurotrophic effect, there is no report on gene expression or serum and CSF production of VEGF in MDD patients. However, there are some reports on cerebral ischemia, Alzheimer's disease and amyotrophic lateral sclerosis. Scheufler et al. (2003) showed an increase in serum and CSF VEGF levels in patients with cerebral ischemia and a significant correlation of CSF VEGF with extensivity of tissue damage. Tarkowski et al. (2002) observed an increase in CSF VEGF levels in patients with Alzheimer's disease and vascular dementia. Nygren et al. (2002) showed that VEGF levels were increased in serum of ALS patients. They discussed that neuronal stress such as oxidative stress or hypoxia could activate expression of VEGF mRNA and production of VEGF in these diseases. Our result also suggests that such stresses may be involved in the pathophysiology of MDD. Our result may be related with previous reports showing an increased expression level of VEGF mRNA in the peripheral monocytes from diabetic patients with coronary artery disease (Panutsopulos et al., 2003). Elevated VEGF production in the serum has been detected in myocardial infarction (Soeki et al., 2000; Ogawa et al., 2000), diabetic retinopathy (Wells et al., 1996), hyperlipidemia (Blann et al., 2001) and hypertension (Belgore et al., 2001). Increased reductive or oxidative stress to the cell, or activation of numerous protein kinase pathways are thought to induces growth factor expression among which the most important is VEGF. There was no patient who was complicated with cardiovascular disease in our study, however, the relationship between MDD and cardiovascular disease is well known. For example, 17% to 27% of patients with coronary artery disease have MDD and the relative risk for the development of coronary artery disease conferred by depression in patients initially free of clinical cardiac disease is approximately 1.5 (Rudisch and Nemeroff, 2003). The elevated VEGF mRNA expression in the leukocytes of depressive patients may reflect a systemic oxidative stress and a risk for cardiovascular disease.

Second, although the VEGF mRNA levels after 8 weeks of paroxetine treatment did not show significant changes from the baseline levels, our result revealed a significant correlation between the VEGF mRNA expression level and the clinical symptoms. The more VEGF mRNA expression after treatment decreased, the more clinical symptoms improved. Our result may suggest that the reduction of systemic stresses such as oxidative stress or hypoxia decreases the VEGF mRNA expression and improve the symptoms of MDD. Although the effects of antidepressant medication on morbidity and mortality in depressed patients after myocardial infarction is controversial (Taylor et al., 2005; Tata et al., 2005), patients with both coronary artery disease and depression have a twofold to threefold increased risk of future cardiac events compared to patients without depression, independent of baseline cardiac dysfunction (Rudisch and Nemeroff, 2003). This may explain why the decrease of VEGF mRNA expression in our study did not correlate with the paroxetine concentration but significantly correlate with clinical improvement.

Third, there were no significant associations between VEGF gene polymorphisms and MDD, although MDD patients showed significantly higher VEGF mRNA expressions than those of controls in our expressional study. The −634G allele and the −2578A allele were associated with lower VEGF production and the risk allele of amyotrophic lateral sclerosis (Lambrechts et al., 2003), Alzheimer's disease (Del Bo et al., 2005) and vascular dementia (Kim et al., 2006). However, VEGF gene polymorphisms do not appear to contribute the pathogenesis of MDD. Since there are racial differences in the distribution of VEGF gene, association analysis in other racial group may be necessary.

In conclusion, our investigation revealed that the mean VEGF mRNA levels were significantly elevated at baseline particularly in MDD patients with first episode and the decrease of VEGF mRNA expression after treatment was positively correlated with the clinical improvement. There were no associations between VEGF gene polymorphisms and MDD or its clinical subtypes. These results suggest that the levels of VEGF mRNA in the leukocytes may be a useful biological marker of MDD. Further studies are necessary to confirm and extend the present results.

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Gene expression in the peripheral leukocytes and association analysis of PDLIM5 gene in schizophrenia

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Abstract

PDLIM5 modulates neuronal calcium signaling, co-localizes with synaptic vesicles of neurotransmitters and positive association between its gene and schizophrenia was reported but its relation is still ambiguous. The differential expression of the PDLIM5 gene both in the brain and in the lymphoblasts has been found in schizophrenia compared to control subjects. In this study, we measured the expression level of the PDLIM5 gene transcripts in the peripheral leukocytes from 19 medication-free and 21 chronically medicated schizophrenic patients as well as age- and sex-matched control subjects using a quantitative real-time PCR method. The mRNA levels of the PDLIM5 gene in the leukocytes of medication-free schizophrenic patients were significantly higher than those of control subjects. On the other hand, our group has previously shown that its mRNA expression in the leukocytes of medication-free major depressive patients was significantly lower compared with controls. There was no difference in the PDLIM5 mRNA levels between chronic schizophrenic patients with antipsychotic medication and their controls. Further, we failed to find any genetic association between the PDLIM5 gene and schizophrenia with six single nucleotide polymorphics (SNPs) of the PDLIM5 gene in Japanese subjects (279 subjects each) and there was no significant relation between PDLIM5 gene and schizophrenia with the haplotype analysis ($P = 0.48$), either. We suggest that the higher expression levels of the PDLIM5 mRNA in the peripheral leukocytes may be a candidate marker for medication-free schizophrenic patients.
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Keywords: PDLIM5; Gene expression; Leukocytes; Association analysis; Schizophrenia

PDLIM5 is an intermediate protein that has been shown to regulate intracellular calcium levels by linking calcium channel and protein kinase C (PKC) [2,3,16]. PDLIM5 is ubiquitously expressed and its cellular localization in the brain is identical to Synapsin which is known to be involved in the neurotransmitter release [16]. The PDLIM5 gene lies on chromosome 4q22, a locus previously reported to be linked with schizophrenia [13,19]. While Kato et al. failed to find any association between the PDLIM5 gene and schizophrenia [15], Horiuchi et al. found a significant association between them [6]. It was reported that the expression level of PDLIM5 mRNA was significantly increased in the postmortem brain tissues of patients with schizophrenia, bipolar disorder and major depression, but was decreased in the immortalized lymphoblastoid cell lines derived from patients with schizophrenia and bipolar disorder [10,11]. Our group has recently shown that levels of mRNA expression in the peripheral leukocytes of the PDLIM5 gene were significantly lower in medication-free major depressive patients compared with controls [8].

The expressional alterations of genes in the peripheral blood lymphocytes and leukocytes have been reported to indicate the changes of the central nervous systems in schizophrenia and

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Table 1a
Demographic data for medication-free schizophrenic patients studied in PDLIM5 mRNA expression analysis ($N = 19$)

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

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

major depressive disorder [7,8,9,17,21]. In this study, we measured the PDLIM5 mRNA expression levels in the peripheral leukocytes in unmedicated and medicated schizophrenic patients as well as in control subjects, using a quantitative real-time PCR method. In addition, we examined the genetic case-control study of the PDLIM5 gene with schizophrenia in Japanese subjects comprising of 279 patients with schizophrenia and 279 controls.

All patients and controls were biologically unrelated Japanese. The diagnosis of schizophrenia was made by at least two experienced psychiatrists according to DSM-IV criteria [1]. Clinical symptoms were evaluated by the Brief Psychiatric Rating Scale scores (BPRS) [20] when blood samples were taken. Age- and sex-matched controls were in good physical health without a history of any psychiatric or serious somatic diseases and taking any medication during the sample collection period. Probands who had first-degree relatives with psychiatric disorders were excluded from the control subjects.

For the measurement of expression levels of the PDLIM5 mRNA, the subjects consisted of 19 medication-free patients with schizophrenia (subject number S1–S19, Tables 1a and 1b) (14 first-episode and drug-naïve schizophrenic patients, 5 schizophrenic patients without antipsychotic treatment for at least 2 months; 9 males and 10 females, mean age: 30.4 ± 9.3), 19 age- and sex-matched controls (9 males and 10 females, mean age: 30.6 ± 8.6), 21 chronically treated patients with schizophrenia who were stably controlled under the same amount dosage of antipsychotics for at least 3 months (subject number S20–S40, Tables 2a and 2b) (13 males and 8 females, mean age: 47.7 ± 11.3) and 21 age- and sex-matched controls (mean age: 47.7 ± 11.1).

For the genetic association study, we used DNA samples from 279 in patients (189 male and 90 female; mean age: 51.3 ± 13.7 years) with schizophrenia from 13 psychiatric hospitals in the neighboring area of Tokushima Prefecture in Japan (population: about 820,000). Age- and sex-matched controls were selected from volunteers after assessing the psychiatric problems (189 male and 90 female; mean age: 51.4 ± 12.0) for the association and haplotype-based case-control study.

All subjects signed written informed consent to participate in the expression and genetic association studies approved by the institutional ethics committees.

Table 1b
PDLIM5 mRNA expression in medication-free schizophrenic ($N = 19$) and control subjects ($N = 19$)

	Male ($N = 9$)	Female ($N = 10$)	Total ($N = 19$)
Schizophrenia (S1–S19)			
Age	28.1 ± 5.6	32.4 ± 11.5	30.4 ± 9.3
The PDLIM5 mRNA expression before treatment	1.13 ± 0.3	1.29 ± 0.3	$1.21 \pm 0.3^{*}$
Control			
Age	27.6 ± 4.8	33.4 ± 10.4	30.6 ± 8.6
The PD1IM5 mRNA expression	0.95 ± 0.2	1.03 ± 0.4	1.00 ± 0.3

The mean PDLIM5 mRNA levels in the peripheral leukocytes from medication-free schizophrenia patients were significantly higher than those of age- and sex-matched controls (Mann–Whitney U test: $P = 0.023$); $^{*}P < 0.05$. No correlation between PDLIM5 mRNA levels and baseline BPRS scores were observed (Spearman's correlation efficient: $P = 0.38$).

Table 2a
Demographic data for chronic schizophrenic patients studied in PDLIM5 mRNA expression analysis ($N=21$)

	Age (y.o)	Gender	Medication	BPRS Score
S20	57	M	QTP 75 mg, LP 150 mg, CP 300 mg	55
S21	56	M	Ris 6 mg	29
S22	56	M	Ris 5 mg, QTP 200 mg, sulpiride 150 mg	44
S23	60	M	Ris 8 mg, LP 20 mg	67
S24	57	M	HPD 9 mg, BPD 9 mg propericyazine 60 mg	52
S25	40	M	Ris 12 mg	33
S26	46	M	Ris 6 mg, HPD 9 mg, sultopride 900 mg	49
S27	45	M	BPD 9 mg, clocaprarmine 75 mg	59
S28	31	M	BPD 2 mg, HPD 1 mg, LP 15 mg Perospirone 24 mg	49
S29	49	F	Ris 6 mg, HPD 6 mg, CP 20 mg, HPD decanoate 150 mg	33
S30	53	F	HPD 2.25 mg, sulpride 150 mg	33
S31	65	F	HPD 4.5 mg, CP 37.5 mg	47
S32	51	F	Olz 10 mg	23
S33	43	F	Ris 6 mg, zotepine 50 mg	45
S34	54	F	Olz 20 mg, LP 50 mg	38
S35	54	M	Ris 12 mg, zotepine 150 mg timiperone 6 mg	42
Sc36	25	M	Ris 9 mg, perospirone 16 mg	39
Sc37	49	M	Ris 12 mg, LP 150 mg	54
Sc38	23	M	Ris 12 mg, LP 150 mg	38
Sc39	35	F	Olz 20 mg	33
Sc40	53	F	QTP 400 mg	27

The age (years old: y.o) represent the age of the subject when the leukocytes were drawn. M: male, F: female, Olz: olanzapine, Ris: risperidone, HPD: hapoperidol, BPD: bromperidol, LP; levom epromazine.

Total RNA was extracted from the peripheral leukocytes using the PAX gene Blood RNA kit (Qiagen, Tokyo, Japan) according to the manufacturer's recommendations. One microgram of total RNA was used for cDNA synthesis by QuantiTect Reverse Transcription Kit (Qiagen) after assessing RNA quality and quantity with NanoDrop (NanoDrop Technologies, DE, USA). Expression of the PDLIM5 gene transcript was quantified by real-time PCR with the TaqMan Gene Expression Assay (Applied Biosystems, CA, USA). Primers and probes (Hs00179051_m1) were purchased from Applied Biosystems as well as Horiuchi's group [6]. GAPDH gene expression was used as an internal control and measurement of threshold cycle (Ct) was performed in triplicate. Data were collected and analyzed with Sequence Detector Software version 2.1 (Applied Biosystems) and the standard curve method. Relative gene expression was calculated as the ratio of PDLIM5 to GAPDH gene and the mean of the three replicate measures was assigned to each individual. Almost all of blood samples were taken in the morning before lunch. The expression of the PDLIM5 mRNA was not changed among blood samples collected at several points during the day time or over several weeks in the same control subjects.

Genotyping was performed using commercially available TaqMan probes (C_2095059_10, C_16015055_20, C_3226622_10, C_16015313_10, C_1569781_10, C_11567561_10) with Applied Biosystems 7500 Fast Real Time PCR System according to the protocol recommended by the manufacturer (Applied Biosystems). We selected six single nucleotide polymorphic (SNP) markers for genotyping according to linkage disequilibrium (LD) and haplotype blocks in the PDLIM5 gene region [6]. Two SNPs (rs10008257, rs2433320) in the 5′-flanking region and four SNPs left in the genomic region are covered about 169-kb in the whole 214-kb of the PDLIM5 gene. The heterozygocities of four of these six SNPs, rs10008257, rs2433320, rs2433327 and rs2452600 in Japanese population are reported as 0.39, 0.18, 0.26 and

Table 2b
PDLIM5 mRNA expression in chronic treated schizophrenic ($N=21$) and control subjects ($N=21$)

	Male ($N=13$)	Female ($N=8$)	Total ($N=21$)
Schizophirenia (S20–S40)			
Age	46.1 ± 12.7	50.4 ± 8.7	47.7 ± 11.3
The PDLIM5 mRNA expression	0.78 ± 0.2	0.93 ± 0.2	0.83 ± 0.2
Control			
Age	46.2 ± 12.3	50.1 ± 9.0	47.7 ± 11.1
The PD1IM5 mRNA expression	0.90 ± 0.3	1.14 ± 0.4	1.00 ± 0.3

The mean PDLIM5 mRNA levels in the peripheral leukocytes from schizophrenia patients who has been treated with antipsychotic drugs for many years were not different from controls' (Mann–Whitney U test: $P=0.16$). No correlation between PDLIM5 mRNA levels and baseline BPRS scores were observed (Spearman's correlation efficient: $P=0.82$).

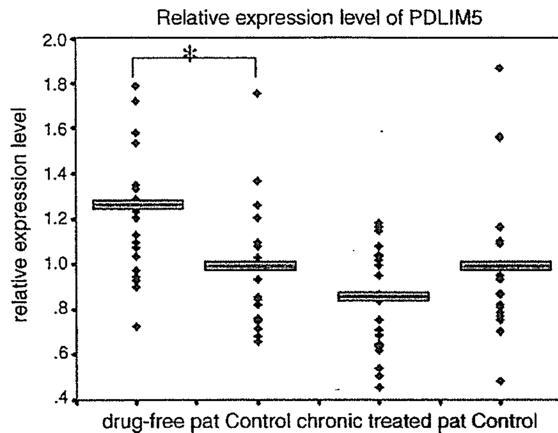

Fig. 1. Relative expression levels of PDLIM5 in the peripheral leukocytes in schizophrenic patients and control subjects. Compared with the normal control group, the mean PDLIM5 mRNA level in the leukocytes of medication-free schizophrenic patients ($N = 19$) was significantly higher (patients: 1.21 ± 0.29, controls: 1.00 ± 0.29, Mann–Whitney U test: $P = 0.023$). The mean PDLIM5 mRNA level in the leukocytes of chronic schizophrenic patients ($N = 21$) showed no significant difference compared with controls (patients: 0.83 ± 0.23, controls: 1.00 ± 0.32, Mann–Whitney U test: $P = 0.16$).

0.34, respectively. The heterozygocities of the other two SNPs, rs12641023 and rs14082, are not reported.

Statistical calculations were carried out using the SPSS Statistical Software Package 11.5 (SPSS, Tokyo, Japan). Expressional differences between patients and control subjects were calculated using the Mann–Whitney U test. Spearman correlation coefficients were used to evaluate the correlations between PDLIM5 mRNA levels and BPRS scores. Two-way ANOVA was performed to determine the independent and combined effects of age and the expression of PDLIM5 between groups. Allele and genotype frequencies of patients and control subjects were compared using Fisher's exact test. The SNPAlyze 3.2 Pro software (DYNACOM, Japan) was used to estimate haplotype frequencies, LD, and permutation P-values. Pair-wise linkage disequilibrium indices, D' and r2, were calculated in the control subjects. The criterion for significance was set at $P < 0.05$ for all tests. Data are presented as mean ± standard deviation.

Relative expression levels of PDLIM5 mRNA in 19 medication-free patients (S1–S19) were 1.21 ± 0.29 in the range of 0.73–1.79, while 1.00 ± 0.29 (range: 0.66–1.75) in healthy volunteers, showing a statistical difference (Mann–Whitney U test: $P = 0.023$, Fig. 1). Mean BPRS scores was 45.2 ± 17.4. No correlation between PDLIM5 mRNA levels and baseline BPRS scores were observed (Spearman's correlation efficient: $P = 0.38$). There was no significant expressional difference of PDLIM5 mRNA levels either between males and females or between genotypes of the single nucleotide polymorphism (rs2433320) both in patients with schizophrenia and in control subjects.

Relative PDLIM5 mRNA level was 0.83 ± 0.23 (0.46–1.18) in 21 chronically treated patients (S20–S40), while 1.00 ± 0.32 (0.49–1.87) in healthy volunteers, showing no significant statistical difference (Mann–Whitney U test: $P = 0.16$; Fig. 1). Mean chlorpromazine-equivalent doses were 932.1 ± 510.5 mg/day and mean duration of treatment was 23.5 ± 10.7 years and mean BPRS scores was 43.1 ± 10.8. No significant relationship between PDLIM5 mRNA levels and BPRS scores was observed (Spearman correlation efficient: $P = 0.71$). There was no significant expressional difference of PDLIM5 mRNA levels either between males and females or between genotypes of the single nucleotide polymorphism (rs2433320) both in patients with schizophrenia and in control subjects.

There were no significant deviations in all six SNPs from Hardy-Weinberg equilibrium in either patients or control subjects. Allele and genotype frequencies of the six SNPs are shown in Table 3. There were no associations between these SNPs and schizophrenia neither in the allelic frequency nor in the genotypic distributions. Although both rs2433320-rs2443327 and rs12641023-rs14082 were in a tight LD ($D' = 0.936$, 0.968, each), permutation test showed no significant difference in estimated frequencies of these haplotypes between the controls and patients (global permutation $P = 0.58$, 0.45, each, Table 4). Haplotypes of six SNPs were evaluated, but no significant difference was observed in frequencies of any estimated haplotype or in distributions of all estimated haplotypes between the controls and patients (global permutation $P = 0.48$).

The present study is the first report on the PDLIM5 gene expression in the peripheral leukocytes in schizophrenia. The mean PDLIM5 mRNA levels in the peripheral leukocytes from medication-free schizophrenia patients were significantly higher than those of age- and sex-matched controls. Altered mRNA expression in the peripheral lymphocytes could reflect the altered metabolism of brain cells [4]. Our result is consistent with the result of higher expression in the postmortem brains from schizophrenic patient but not with the result of lower expression in the lymphoblastoid cells derived from schizophrenic patients [10,11]. The differences of the mRNA expression between studies may be partly attributed to the difference in the materials. When using lymphoblastoid cells, the effect of virus infection or chromosomal alterations during culture must be taken into account [12]. On the other hand, the mRNA expression level of PDLIM5 gene was not significantly higher in chronically treated schizophrenics compared with that of controls. This finding in the chronic patients may be a consequence of pharmacological effects of antipsychotics or clinical improvement. This result suggests that expression of PDLIM5 mRNA may not be trait-oriented but state-related change. To confirm whether the expression of this gene is a state marker, a follow-up investigation is needed in the same patients before and after treatment.

The pathophysiological mechanism remains unknown, but we speculate that the higher expression of PDLIM5 is related with putatively elevated Ca^{2+} signaling in schizophrenia. It has been suggested that abnormalities in Ca^{2+} signaling was associated with molecular etiology of schizophrenia. Regulator of G protein signaling-4 (RGS4) and B-cell limphoma/leukaemia-2 gene (Bcl-2) which reduce free Ca^{2+} in a cell have been found to be down regulated in the temporal cortex of schizophrenic patients [14,18]. It was reported that there was high levels of free intracellular Ca^{2+} in platelets of schizophrenic patients

Table 3
Genetic studies of PDLIM5 gene with schizophrenia in case-control samples

Group	Genotype			*n*	Hardy-Weinberg equilibrium	*P*-value	Allele		*P*-value
ra1 0008257	A/A	A/G	G/G				A	G	
sch	42	127	105	274	0.823	0.471	211	337	0.804
cont	34	140	102	276	0.229		208	344	
rs2433320									
sch	7	75	197	279	0.858	0.601	89	469	0.871
cont	11	70	198	279	0.205		92	466	
rs2433327	T/T	T/C	C/C				T	C	0.833
sch	169	88	16	273	0.414	0.917	426	120	
cont	164	92	15	271	0.788		420	122	
rs2452600	T/T	T/C	C/C				T	C	
sch	54	125	96	275	0.306	0.232	233	317	0.080
cont	68	130	81	279	0.325		266	292	
rs12641023	A/A	A/G	G/G				A	G	
sch	51	126	93	270	0.555	0.497	228	312	0.295
cont	42	131	103	276	0.924		215	337	
rs14082	A/A	A/G	G/G				A	G	
sch	58	124	91	273	0.243	0.302	240	306	0.141
cont	45	125	103	273	0.582		215	331	

sch: Schizophrenia, cont: control subjects. *P*-values are calculated by Fisher s exact test.

[22]. PDLIM5 regulates intracellular calcium levels by linking calcium channel and protein kinase C [2,3,16]. The levels of PDLIM5 might be up-regulated both in the brain and in the peripheral leukocytes in patients with schizophrenia in response to increased intracellular calcium levels. It has been demonstrated that antipsychotic drugs block IP3-induced release of Ca^{2+} [23] and Ca^{2+} dependence of PKC is well known [5]. So antipsychotic medication might normalize the up-regulation of PDLIM5 expression in schizophrenia by reducing Ca^{2+} signaling.

PDLIM5 may be involved in other mental disorders. Iwamoto et al. reported that expression level of PDLIM5 was significantly and commonly increased in the postmortem brain tissues of patients with schizophrenia, major depression and bipolar disorder [11]. However, we have already shown that mean PDLIM5 mRNA level in the peripheral leukocytes of medication-free patients with major depression was significantly lower than in control subjects [8]. Therefore, the higher expression of this gene in the peripheral leukocytes of medication-free patients with schizophrenia may be disease-specific and not due to non-specific stress of psychiatric condition. Further investigations of other psychiatric diseases including bipolar disorder are needed.

Table 4
Linkage disequilibrium (LD) indices (lower left are r2, upper right are *D′*)

	rs10008257	rs2433320	rs2443327	rs2452600	rs12641023	rs14082
rs10008257		0.37227	0.44147	0.28294	0.12734	0.15919
rs2433320	0.01632		0.9364	0.50709	0.37209	0.40839
rs2443327	0.03427	0.57719		0.54423	0.43945	0.45693
rs2452600	0.0447	0.05573	0.09541		0.19114	0.18089
rs12641023	0.00626	0.04284	0.08854	0.02152		0.96845
rs14082	0.01002	0.05068	0.09508	0.01918	0.93062	

Horiuchi et al. reported that there were significant association between polymorphisms (rs2433320 and rs2433322) of PDLIM5 gene and schizophrenia. Their group also showed that the different alleles of the rs2433320 showed different DNA–protein complexes on electrophoreic mobility shift assay and GA heterozygotic genotype might have higher transcriptional activity in schizophrenia [6]. However, our result showed that there was not significant association between schizophrenia and six polymorphisms of PDLIM5 gene, including rs2433320, and this result is consistent with a previous study with a large number of subjects ($n = 562$) [15]. In addition, neither patients nor controls showed a significant difference of the PDLIM5 mRNA expression in the peripheral leukocytes between GG and GA genotypes of this SNP in our subjects although the typeII error was not denied.

In conclusion, our investigation revealed that the mean PDLIM5 mRNA levels in medication-free schizophrenic patients were significantly higher compared to those in controls and the chronic schizophrenic patients with antipsychotic treatment for many years showed almost the same expression levels as healthy control levels. There were no associations between schizophrenia and PDLIM5 gene. These results suggest that the higher expression levels of PDLIM5 mRNA in the leukocytes may be a candidate marker for medication-free schizophrenic patients. Further studies are necessary to confirm the present results.

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Impact of the DISC1 Ser704Cys polymorphism on risk for major depression, brain morphology and ERK signaling

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Disrupted-in-schizophrenia 1 (DISC1), identified in a pedigree with a familial psychosis with the chromosome translocation (1:11), is a putative susceptibility gene for psychoses such as schizophrenia and bipolar disorder. Although there are a number of patients with major depressive disorder (MDD) in the family members with the chromosome translocation, the possible association with MDD has not yet been studied. We therefore performed an association study of the DISC1 gene with MDD and schizophrenia. We found that Cys704 allele of the Ser704Cys single-nucleotide polymorphism (SNP) was associated with an increased risk of developing MDD ($P = 0.005$, odds ratio = 1.46) and stronger evidence for association in a multi-marker haplotype analysis containing this SNP ($P = 0.002$). We also explored possible impact of Ser704Cys on brain morphology in healthy volunteers using MR imaging. We found a reduction in gray matter volume in cingulate cortex and a decreased fractional anisotropy in prefrontal white matter of individuals carrying the Cys704 allele compared with Ser/Ser704 subjects. In primary neuronal culture, knockdown of endogenous DISC1 protein by small interfering RNA resulted in the suppression of phosphorylation of ERK and Akt, whose signaling pathways are implicated in MDD. When effects of sDISC1 (Ser704) and cDISC1 (Cys704) proteins were examined separately, phosphorylation of ERK was greater in sDISC1 compared with cDISC1. A possible biological mechanism of MDD might be implicated by these convergent data that Cys704 DISC1 is associated with the lower biological activity on ERK signaling, reduced brain gray matter volume and an increased risk for MDD.

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INTRODUCTION

The disrupted-in-schizophrenia 1 (DISC1) gene was initially identified at the breakpoint of a balanced translocation (1,11)(q42.1;q14.3), which segregated with major mental disorders in a large Scottish family (1). In this family, patients with schizophrenia, bipolar disorder and recurrent major depressive disorder (MDD) had been identified as carriers of the translocation (1,2). Subsequent genetic studies in several independent populations, including association and linkage studies, have also suggested that the DISC1 gene may be implicated in both schizophrenia and bipolar disorder (3–10). Previous studies suggest that genetic variations within the DISC1 gene, which increase risk for schizophrenia, are associated with the cerebral cortical gray matter and hippocampal volumes and function (9,11,12). However, no association study between the DISC1 gene and MDD has not yet been reported, despite there are more patients with MDD than those with schizophrenia in the original Scottish family members with translocation.

DISC1 is a multi-functional protein. Several research groups have identified DISC1 interacting proteins, which are associated with the components of the cytoskeleton and centrosome, such as dynein, Nudel, elongation protein zeta-1, etc. (13–18). DISC1 plays critical roles on the cerebral cortex development via microtubular dynamics and the DISC1–dynein complex (13). Another function of DISC1 may be modulation of cAMP signaling via an interaction with phosphodiesterase 4B, which also has been found to be disrupted by a balanced translocation in a patient with schizophrenia (19). Other functions of DISC1, including the mitochondrial and nuclear related functions, have also been suggested (15,20,21).

Here, we report an association of the genetic variations of DISC1, including Ser704Cys (rs821616) single-nucleotide polymorphism (SNP) and MDD. The risk allele (Cys704) for MDD is associated with the reduced gray matter volume and fractional anisotropy (FA) in prefrontal white matter in healthy subjects. Furthermore, the risk allele is linked to lower ERK activity (extracellular signal-regulated kinase), which has been suggested to have a role in the pathophysiology in MDD.

RESULTS

Association between the genetic variants of the DISC1 gene and major depression

We examined possible association between genetic variants in the DISC1 gene, including Ser704Cys SNP and MDD or schizophrenia. We genotyped 13 SNPs to cover the DISC1 locus at an average density of 31 kb. We found a significant association between genetic variations in DISC1 and MDD (Table 1). The Cys allele frequency of SNP12 (Ser704Cys) was greater in patients with MDD when compared with controls ($\chi^2 = 7.88$, df $= 1$, $P = 0.005$, odds ratio $= 1.46$, 95% CI 1.12–1.92). There was a weak evidence for an association with SNP1 ($P = 0.048$). As previous association studies of the DISC1 gene applied three-marker haplotype analysis (4,8,9), we performed this analysis. Consistent with the individual marker results, the three marker haplotypes containing SNPs 11–13 were associated with MDD: SNP11 (G allele)–SNP12(Cys)–SNP13(C allele) ($P = 0.002$; Table 2). When we examined the association between genetic variations of DISC1 and schizophrenia, a weak evidence for association was observed with SNP7 ($P = 0.0496$), whereas SNP12 (Ser704Cys) was not significantly associated with schizophrenia (Table 1). Because a prior study reported sex-dependent effects on association with schizophrenia (4), we examined males and females separately. This analysis revealed stronger evidence for association with SNP9 in female patients with schizophrenia ($P = 0.0088$), but no association in male subjects, whereas a prominent gender effect was not found in MDD (Table 3). Three marker haplotypes were not associated with schizophrenia (data not shown). SNP12 showed a strong linkage disequilibrium (LD) with SNP10 and SNP11, moderate LD with SNP1 and SNP13 and weak LD with SNP6 in controls, and similar LD results were obtained in MDD and schizophrenia (Supplementary Material, Tables S1–S3). LD pattern of our data was similar to that of HapMap database in the Japanese population. Our results suggest that DISC1 is associated with MDD and with schizophrenia and that Ser704Cys SNP, in particular, is associated with MDD in our sample.

Effects of the DISC1 Ser704Cys polymorphism on *in vivo* brain structure

As the abnormalities in the brain gray matter volume and white matter microstructure have been implicated in the biology of mood disorders and schizophrenia (22,23), we examined the possible effects of the Ser704Cys SNPs and other associated SNPs with schizophrenia on brain structure in healthy subjects (demographic information for Ser704Cys, Table 4). We found a bilateral (left dominant) reduction in the gray matter volume in the anterior cingulate cortex (ACC) [Brodmann area (BA) 24, Talairach coordinates $x, y, z = -6, 27, 17$, respectively, $t = 3.58$], cingulate gyrus (BA24, Talairach coordinates $x, y, z = 0, 3, 34$, respectively, $t = 3.7$) and the posterior cingulate gyrus (BA31, Talairach coordinates $x, y, z = -9, -39, 36$, respectively, $t = 3.39$) in cys-DISC1 carriers (cys/cys and cys/ser) compared with ser/ser-DISC1 individuals by tensor-based morphometry (TBM) analysis (Fig. 1, upper panel). When we analyzed the effect of this SNP on brain morphology in the other direction (i.e. cys > ser), we found a significant reduction in the volumes of the lateral ventricle, interhemispheric fissure and the bilateral Sylvian fissure in ser/ser-DISC1 individuals compared with cys-DISC1 carriers (Fig. 1, lower panel). This finding suggests that cys-DISC1 carriers have expanded cerebrospinal fluid (CSF) space compared with ser/ser-DISC1 individuals, possibly consistent with our finding that cys carriers in our analysis have reductions in tissue volume measures. We did not detect significant differences in hippocampal volume related to this DISC1 polymorphism with even at a lenient threshold at $P < 0.05$. In comparison with ser/ser-DISC1 individuals, cys-DISC1 carriers demonstrated a significantly decreased FA value in the frontal white matter ($P < 0.001$) (Fig. 2). The reversed contrast, i.e. increased FA in cys-DISC1 carriers, was not observed even at the level of

Table 1. Allele distributions for 13 SNPs in the DISCI gene in patients with MDD, those with schizophrenia and controls

SNP	dbSNP	Distance from SNPI	Major/minor polymorphism	Amino acid substitution	Controls $n = 717$	MDD $n = 373$	P-value	OR	SZ $n = 658$	P-value	OR
1	rs6541281		C/T		0.122	0.094	*0.048*	*0.75*	0.105	0.16	—
2	rs3738401	17161	G/A	Gln264Arg	0.260	0.263	0.89	—	0.264	0.83	—
3	rs1340982	48898	C/T		0.461	0.456	0.82	—	0.459	0.92	—
4	rs1322784	115801	T/C		0.351	0.371	0.36	—	0.375	0.19	—
5	rs1535529	141475	C/T		0.425	0.441	0.48	—	0.410	0.42	—
6	rs7551537	159083	C/T		0.363	0.363	0.98	—	0.382	0.29	—
7	rs999710	197809	C/T		0.404	0.424	0.39	—	0.441	*0.05*	1.16
8	rs967433	218681	T/C		0.409	0.416	0.76	—	0.387	0.24	—
9	rs821577	253923	G/T		0.201	0.166	0.05	—	0.172	0.06	—
10	rs821597	289130	T/C		0.414	0.438	0.28	—	0.404	0.60	—
11	rs843979	317474	C/G		0.335	0.358	0.29	—	0.334	0.92	—
12	rs821616	331464	A/T	Ser704Cys	0.104	0.145	*0.005*	*1.46*	0.122	0.14	—
13	rs2806465	353160	G/C		0.376	0.378	0.92	—	0.370	0.75	—

Minor allele frequencies in controls are shown. SZ, schizophrenia, OR, odds ratio. Significant results ($P < 0.05$) indicated by italics.

Table 2. Three-marker haplotype analysis among patients with major depression and controls

SNP	Haplotype										
1	2										
2	1	1									
3	1	1	2								
4		2	1	1							
5			2	1	1						
6				2	2	2					
7					1	1	1				
8						2	2	1			
9							2	2	2		
10								1	2	2	
11									1	2	2
12										2	2
13											2
Global P-value	*0.030*	0.19	*0.043*	0.81	0.74	0.55	0.69	0.20	*0.012*	0.17	0.057
Individual P-value	*0.017*	*0.026*	*0.018*	0.24	0.25	0.25	0.19	*0.023*	*0.019*	*0.017*	*0.002*
Haplotype frequency in control	0.050	0.201	0.180	0.123	0.136	0.108	0.081	0.064	0.018	0.096	0.074
Haplotype fequency in MDD	0.026	0.250	0.233	0.104	0.116	0.089	0.060	0.035	0.000	0.130	0.116

Major allele = 1, minor allele = 2. Individual P-value indicates the best P-value among the haplotypes. Significant results ($P < 0.05$) indicated by italics.

$P < 0.05$. Our results suggest that the Ser704Cys SNP of the DISC1 gene might affect human gray matter volume and white matter microstructure, particularly in peri-cingulate area. On the other hand, genotype effects of other SNPs, i.e. SNP1, SNP7 and SNP9 on the brain morphology or white matter microstructure, were not detected.

DISC1 and ERK signaling

ERK signaling and Akt signaling have been implicated in MDD (24,25). Thus, we investigated the possible involvement of DISC1 on ERK and Akt signaling pathways. The endogenous DISC1 function in cortical cultures was examined using small interfering RNA (siRNA) for DISC1, and robust decrease (70%) of endogenous DISC1 protein was confirmed (Fig. 3Aa and d). We found decreased levels of phosphorylation of ERK1/2 (pERK1/2: an activated form of ERK) and phosphorylation of Akt (pAkt: an activated form of Akt) after DISC1-siRNA transfection, suggesting that endogenous DISC1 protein is involved in ERK and Akt activations (Fig. 3Aa–c). In contrast, the levels of total ERK1/2, Akt and TUJ1 (class III β-tubulin, a neuronal marker) proteins were not altered (Fig. 3Aa, quantified data (mean ± SD): ERK1: 1.15 ± 0.22, 0.95 ± 0.27; ERK2: 1.01 ± 0.17, 0.99 ± 0.22; Akt: 1.05 ± 0.06, 1.09 ± 0.04; TUJ1: 1.03 ± 0.07, 1.01 ± 0.07; relative to none; scramble, si-DISC1, respectively).

To examine the effect of Ser704Cys SNP on ERK and Akt signaling, we overexpressed each type of DISC1 protein (sDISC1: DISC1 protein with Ser704; cDISC1: DISC1 protein with Cys704) using a sindbis virus-mediated gene delivery system. Neuronal cultures infected with the control viral construct [green fluorescence protein (GFP) only] were doubly stained with GFP signal and immunostaining signal

Table 3. Gender difference of allele distributions for 13 SNPs in the DISCI gene among patients with schizophrenia, those with major depression and controls

SNP female								Male						
	Control	SZ			MDD			Control	SZ			MDD		
	$n = 366$	$n = 318$	*P*-value	OR	$n = 226$	*P*-value	OR	$n = 351$	$n = 340$	*P*-value	OR	$n = 147$	*P*-value	OR
1	0.135	0.102	0.061	—	0.091	*0.021*	*0.64*	0.108	0.107	0.96	—	0.099	0.65	—
2	0.258	0.274	0.52	—	0.279	0.44	—	0.262	0.254	0.74	—	0.238	0.43	—
3	0.466	0.459	0.80	—	0.465	0.97	—	0.456	0.459	0.91	—	0.442	0.69	—
4	0.344	0.385	0.12	—	0.356	0.68	—	0.359	0.366	0.78	—	0.395	0.29	—
5	0.414	0.399	0.58	—	0.469	0.063	—	0.437	0.421	0.53	—	0.398	0.25	—
6	0.373	0.381	0.77	—	0.354	0.51	—	0.352	0.384	0.22	—	0.378	0.44	—
7	0.404	0.447	0.12	—	0.427	0.44	—	0.405	0.437	0.23	—	0.418	0.69	—
8	0.411	0.390	0.42	—	0.425	0.65	—	0.406	0.384	0.40	—	0.401	0.89	—
9	0.209	0.154	*0.0088*	*1.45*	0.164	0.054	—	0.192	0.190	0.90	—	0.170	0.41	—
10	0.399	0.426	0.31	—	0.442	0.14	—	0.430	0.384	0.08	—	0.432	0.96	—
11	0.328	0.343	0.56	—	0.369	0.14	—	0.343	0.325	0.47	—	0.340	0.92	—
12	0.111	0.137	0.14	—	0.146	0.073	—	0.097	0.107	0.52	—	0.143	*0.035*	*0.64*
13	0.383	0.363	0.46	—	0.363	0.50	—	0.369	0.376	0.77	—	0.401	0.34	—

Minor allele frequencies in controls are shown. SZ, schizophrenia; OR, odds ratio. Significant results ($P < 0.05$) indicated by italics.

Table 4. Demographic information for the brain MRI study

Variables	ser/ser-DISC1 ($n = 86$)	cys-DISC1 carriers ($n = 22$)	*P*-value
Age	35.1 (11.5)	40.1 (13.8)	0.08
Gender (M/F)	33/53	7/15	0.81
Education years	16.7 (2.8)	16.8 (3.4)	0.89
Full-scale IQ	111.8 (11.5)	109.2 (13.7)	0.39

Cys-DISC1 carriers ($n = 22$): cys/ser-DISC1 ($n = 21$) and cys/cys-DISC1($n = 1$). Mean values (SD) are presented.

by anti-microtubule-associated protein 2 (MAP2: a neuronal dendritic marker) antibody, as well as viral constructs of two types of DISC1 (sDISC1 and cDISC1) (Fig. 3Ba). About 85% of MAP2-positive cells in control-, sDISC1- or cDISC1-infected cortical cultures were GFP positive, indicating that the majority of neurons were infected, respectively [control (mean ± SD): 84.4 ± 6.4%; sDISC1: 86.6 ± 5.1%; cDISC1: 86.3 ± 9.8%, $n=6$ fields, selected randomly]. When cortical cultures infected by sDISC1 or cDISC1 were doubly stained with GFP signal and immunostaining signal by the DISC1 antibody, the sDISC1 and cDISC1 proteins were located in both cell body and neurites in punctuate manner, especially perinuclear region and neurite branch (Fig. 3Bb). As expression levels of GFP and TUJ1 were not altered by viral infections and both sDISC1- and cDISC1-infected cultures showed similar levels of DISC1 expression (Fig. 3Ca, quantified data (mean ± SD): GFP: 1.00 ± 0.10, 0.92 ± 0.15; TUJ1: 0.98 ± 0.07, 0.98 ± 0.09; DISC1: 5.43 ± 0.52, 5.43 ± 0.33, relative to control, sDISC1, cDISC1, respectively), we examined the phosphorylation of ERK and Akt in this system. Two-way ANOVA indicated significant main effects of viral infection ($F = 202$, $df = 2$, $P < 0.001$) and measurement of protein levels ($F = 161$, $df = 8$, $P < 0.001$) and interaction of these two factors ($F = 44.4$, $df = 16$, $P < 0.001$). There were significant effects of viral infection in the measurements of pERK1/2 and pAkt; however, neither total ERK1/2 nor Akt was altered after virus infection (pERK1: $F = 104$, $P < 0.001$; pERK2: $F = 29.2$, $P < 0.001$; pAkt: $F = 9.0$, $P < 0.01$; ERK1: $F = 1.8$, $P > 0.2$; ERK2: $F = 3.0$, $P > 0.1$; Akt: $F = 0.2$, $P > 0.8$) (Fig. 3C). *Post hoc* comparison revealed significant increases in pERK1/2 in sDISC1- or cDISC1-infected cultures compared with control (pERK1: sDISC1 $P < 0.01$; cDISC1 $P < 0.01$; pERK2: sDISC1 $P < 0.05$; cDISC1 $P < 0.05$) (Fig. 3Ca and b). The levels of ERK1 activation in sDISC1-overexpressing cultures were more intensive than those in cDISC1-overexpressing cultures ($P < 0.01$) (Fig. 3Ca and b). A significant elevation of pAkt was observed in the sDISC1-overexpressing cultures compared with control ($P < 0.05$), although an increase in the pAkt level by cDISC1 overexpression was not significant ($P > 0.1$) (Fig. 3Ca and c). However, there were no significant difference of pAkt levels between sDISC1 and cDISC1 ($P > 0.1$).

We next examined rescue experiments, transfection with sDISC1 or cDISC1 into primary culture knocked down to DISC1. In this experiment, siRNA for DISC1 decreased phosphorylation of ERK by ~50% and there were significant effects of viral infection in the measurements of pERK1/2 (pERK1: $F = 7.3$, $P < 0.01$; pERK2: $F = 9.4$, $P < 0.01$) (Fig. 3D). *Post hoc* comparison revealed a significant elevation of pERK1 levels in the sDISC1-overexpressing cultures compared with si-DISC1 treatment ($P < 0.01$), although an increase in the pERK1 level by cDISC1 overexpression was not statistically significant ($P > 0.1$) (Fig. 3D). A significant elevation of pERK2 was observed in sDISC1- and cDISC1-infected cultures compared with si-DISC1 treatment (sDISC1: $P < 0.05$; sDISC1: $P < 0.05$) (Fig. 3D). Western blots showed that total ERK, Akt and TUJ1 protein levels were not changed and that pAkt levels were also rescued by DISC1 overexpression (Fig. 3Da). These results suggest the recovery of the activation of ERK1/2 and Akt after sDISC1- and cDISC-overexpression in DISC1 knockdown cultures. The effect of rescue on phosphorylation of ERK was larger in sDISC1 compared with cDISC1, although the difference did not reach the statistical significance (Fig. 3D). This

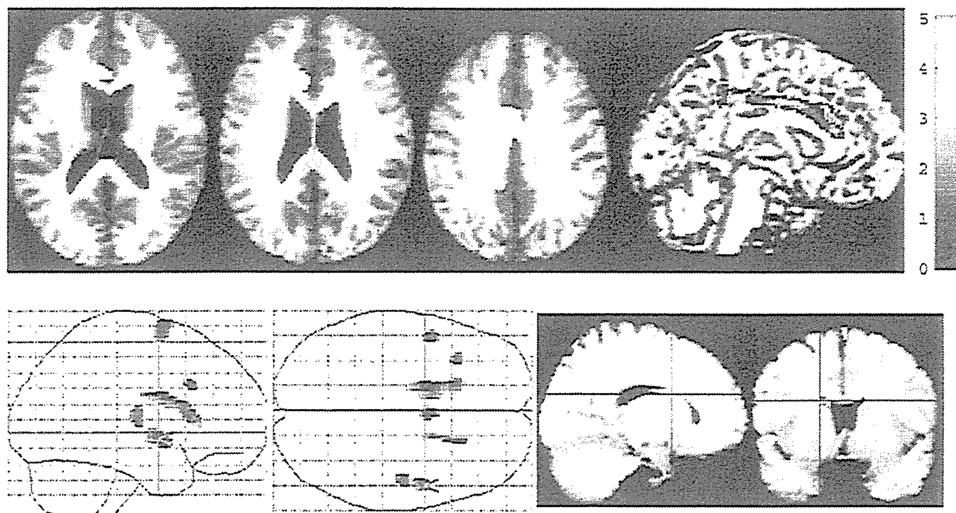

Figure 1. Impact on the brain morphology of the Ser704Cys SNP in healthy subjects. (Upper panel) The SPM {t} is displayed onto T1-weighted MR images. Cys-DISC1 carriers ($n = 22$) had reduced volumes in the bilateral ACC, cingulate gyrus and the posterior cingulate gyrus compared with ser/ser-DISC1 individuals ($n = 86$). (Lower panel) The SPM{t} is displayed on a standard maximum intensity projection images and T1-weighted MR images. Ser/Ser-DISC1 individuals demonstrated decreased volumes of the lateral ventricle, interhemispheric fissure and bilateral Sylvian fissure, indicating an expansion of the CSF space in cys-DISC1 carriers.

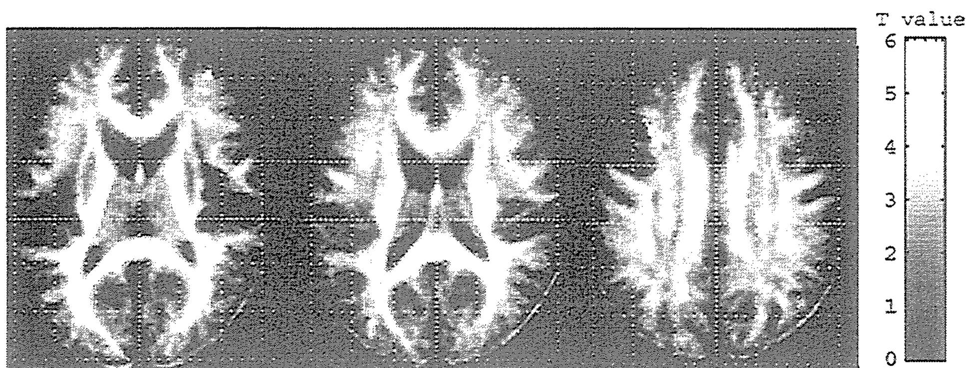

Figure 2. Disruption of white matter integrity revealed by DTI. The SPM{t} is displayed onto a FA map. A significant reduction in FA in the prefrontal white matter was found in the cys-DISC1 carriers ($n = 22$) when compared with individuals with ser/ser-DISC1 ($n = 86$).

might be due to the complexity of this experiment (siRNA plus overexpression) compared with the overexpression only experiments, as the standard deviations of these experiments were larger than those of overexpression experiments. Our results suggest a possible role of DISC1 in the ERK and Akt signaling and an impact of Ser704Cys on ERK activation.

DISCUSSION

Here, we report the evidence for association between MDD and the Ser704Cys SNP as well as several haplotypes including the Ser704Cys SNP. We also replicated earlier evidence for a weak association with schizophrenia which is stronger in female patients (4). Previous association studies with schizophrenia suggested several regions in the DISC1 gene, such as intron2–intron3, intron4–intron9 and intron9–exon13 (4, 5, 8, 9). The region of intron8–intron9 is common across the studies. Consistent with the previous studies, two SNPs in intron9 were associated with schizophrenia in our study. Both false-positive and false-negative associations due to population stratification cannot be excluded in our case–control study, despite the precaution of ethnic matching of this study. Differences in gender ratio and ages between groups could be potential confounding factors. Therefore, it is necessary to carry out further investigations to confirm our findings in other samples. It has also suggested that allelic heterogeneity exists for association between the DISC1 gene and psychiatric illness (26), and this may explain different alleles being associated with illness in our sample compared with others.

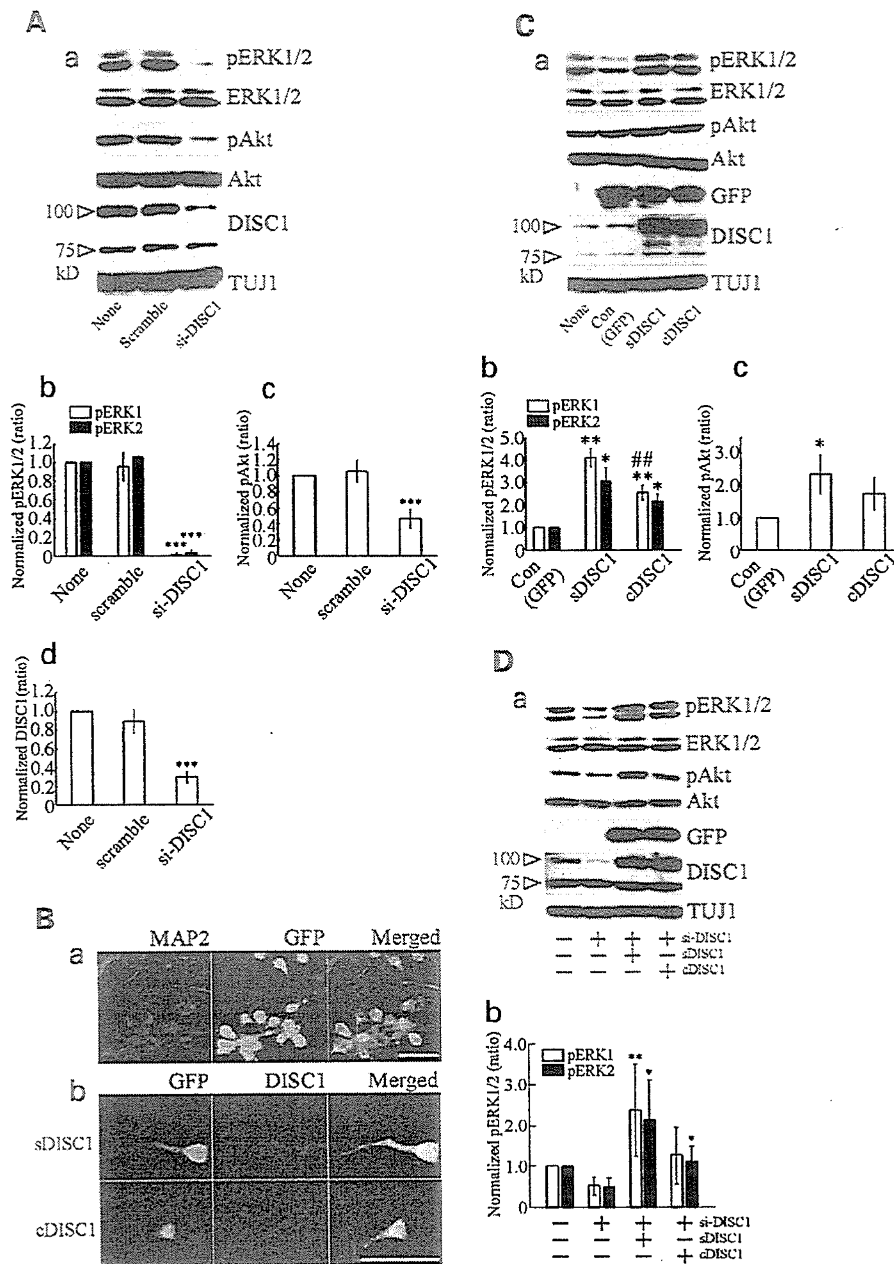

Figure 3. Effects of the DISC1 protein on the ERK and Akt signaling in cortical neurons. (**A**) Suppression of phosphorylation of ERK and Akt in DISC1-siRNA-transfected cultures. Cortical cultures after DIV4 were treated with siRNA for DISC1 (si-DISC1; 100 nM) or control (scramble; 100 nM) for 72 h. Cortical cultures were harvested at DIV7 for western blotting for pERK1/2, ERK1/2, pAkt, Akt, DISC1 or TUJ1. The immunoblots shown are representative of four independent experiments (a). Quantification of the immunoreactivity of pERK1/2 (b), pAkt (c) or DISC1 (d). Quantitative data represent the mean ± SD ($n = 4$). $^{***}P < 0.001$ versus scramble. (**B**) (a) Double staining with GFP (green) signal and immunostaining signal by anti-MAP2 (red, a neuronal marker) antibody after sindbis virus-mediated gene transfer. Representative control (GFP only)-infected cortical cultures were shown. (b) GFP and DISC1 signal after sDISC1 (*upper*) or cDISC1 (*lower*) gene transfer, respectively. DISC1 localization was detected as a red signal. Virus infection was performed at DIV4 and infected cultures were fixed at DIV6 for immunostaining. Bar = 50 µm. (**C**) Differential activation of ERK and Akt between sDISC1 and cDSIC1. Samples for blotting pERK1/2, ERK1/2, pAkt, Akt, GFP, DISC1 or TUJ1 were prepared 24 h (DIV5) after viral infection at DIV 4 (a). The quantification of pERK1/2 (b) or pAkt (c) levels after overexpression of sDISC1 or cDISC1 was shown. The immunoblots shown are representative of four independent experiments. Quantitative data represent the mean ± SD ($n = 4$). $^{**}P < 0.01$, $^{*}P < 0.05$ versus control, ## $P < 0.001$ versus sDISC1. (**D**) (a) Recovery of the activation of ERK1/2 and Akt after sDISC1 and cDISC overexpressing in DISC1 knockdown cultures. To downregulate endogenous DISC1, si-DISC1 was applied at DIV4 or DIV5 cultures. Sindbis virus-infection for sDISC1 or cDISC1 overexpression was performed 48 h after the si-DISC1 treatment. Samples for blotting for pERK1/2, ERK1/2, pAkt, Akt, GFP, DISC1 or TUJ1 were prepared 48 h after viral infection. The immunoblot images are representative of five independent experiments. (b) The quantification of pERK1/2 for each experimental condition was shown. Quantitative data represent the mean ± SD ($n = 5$). $^{**}P < 0.01$, $^{*}P < 0.05$ versus si-DISC1.

We demonstrated that healthy subjects with the risk allele carriers for MDD (cys-DISC1) had relatively reduced the gray matter volumes in cingulate cortex, relatively expanded CSF space and reduced the FA values in the prefrontal white matter. This pattern of changes on magnetic resonance imaging (MRI) scanning, specifically the gray matter volume deficits in the ACC, expanded the CSF and reduced the FA values in prefrontal cortex, has been repeatedly reported in the studies of patients with schizophrenia and MDD (22,27–29). Several studies demonstrated a decreased volume in the ACC in patients with MDD in remission, MDD with a family history or in early onset depression (30–33) and abnormalities of cortical neuronal organization in postmortem brain of MDD have been reported in the ACC (34). It has been reported that relatively higher FA is associated with remission of MDD, following treatment with drugs or electroconvulsive therapy; however, reduced prefrontal FA has not been reported consistently in MDD (29,35–37). These various findings suggest that decreased gray matter volume and FA in the frontal area might be associated with the increased risk for MDD. Previous studies found that the risk haplotype of the DISC1 gene affected cortical gray matter and that Ser704Cys SNP had an impact on the hippocampal structure and function (9,11); however, we did not observe either effects of SNPs associated with schizophrenia in our sample on cortical gray matter or effects of Ser704Cys SNP on hippocampal volume. Moreover, in our study of the effect of Ser/Cys genotype on brain imaging derived phenotypes and clinical association, it is the cys allele that is relatively deleterious, whereas in an earlier study, it was the ser allele (9). These inconsistencies may relate to sample differences, methodological differences, and also to possible genetic and allelic heterogeneity.

We found robust effects of DISC1 on ERK and Akt signaling and evidence that the cDISC1 (the risk allele for MDD) might exert a weaker effect on the ERK activation than sDISC1. The involvement of ERK in the therapeutic mechanisms of mood disorder has been proposed (38,39). It has been shown that ERK can phosphorylate PDE4 and alter its activity (40,41) and that PDE inhibitors might have antidepressant efficacy (24). Taken together, the regulation of ERK signaling by DISC1 may contribute, at least in part, to the mechanisms of the risk for MDD. Structural imaging studies have demonstrated reduced gray matter volumes and white matter abnormality in several brain areas of patients with mood disorders relative to healthy controls, and postmortem morphometric brain studies also demonstrated cellular atrophy and/or loss (24). As the ERK kinase signaling is implicated in cytoskeletal remodeling, neurite outgrowth and cell survival (24) and decreased expression of ERK was observed in the postmortem brain of depressive patients (42), impaired ERK signaling could be related to the structural abnormality in major depression.

In conclusion, we have found evidence for association between genetic variation of DISC1 and MDD, brain morphology and ERK signaling pathway. Our data suggest that Ser704Cys might be a functional variant that impacts on neural mechanisms implicated in the biology of major depression.

MATERIALS AND METHODS

Subjects

Subjects for the clinical association study were recruited at Fujita Health University School of Medicine, Showa University School of Medicine and National Center of Neurology and Psychiatry, Japan. They were 373 patients with MDD [147 males and 226 females with mean age of 54.0 years (SD 16.0); mean age of onset of 46.5 years (SD 15.3)], 658 patients with schizophrenia [340 males and 318 females with mean age of 43.6 years (SD 14.6); mean age of onset of 24.2 years (SD 8.6)] and 717 healthy comparison subjects [351 males and 366 females with mean age of 41.3 years (SD 16.9)]. All the subjects were Japanese. Consensus diagnosis was made for each patient by at least two psychiatrists, according to the Diagnostic and Statistical Manual of Mental Disorders, 4th edition (DSM-IV Criteria). Control subjects were healthy volunteers who had no current or past contact to psychiatric services.

One hundred and eight healthy Japanese (biologically unrelated) for MR experiments were recruited at the National Center of Neurology and Psychiatry and screened by a questionnaire on medical history and excluded if they had neurological, psychiatric or medical conditions that could potentially affect the central nervous system, such as substance abuse or dependence, atypical headache, head trauma with loss of consciousness, asymptomatic or symptomatic cerebral infarction detected by the T2-weighted MRI, hypertension, chronic lung disease, kidney disease, chronic hepatic disease, cancer or diabetes mellitus. Detail demographics of subjects in genotypes of SNP1, SNP7, SNP9 and SNP12 (Ser704Cys) were noted in Supplementary Material. After description of the study, written informed consent was obtained from every subject. The study protocol was approved by institutional Ethics Committees.

Genetic analysis

Venous blood was drawn from subjects and genomic DNA was extracted from the whole blood according to the standard procedures. Thirteen SNPs were genotyped using the TaqMan 5′-exonuclease allelic discrimination assay as described previously (43,44). Primers and probes for detection of the SNPs are available upon request. Statistical analysis of association studies was performed using SNPAlyse (DYNACOM, Yokohama, Japan). Allele distributions between patients and controls were analyzed by the χ^2 test for independence. The measure of LD, denoted as D′ and r^2, was calculated from the haplotype frequency using the expectation–maximization algorithm. Case–control haplotype analysis was performed by the permutation method to obtain the empirical significance (45). The global P-values represent the overall significance using the χ^2 test when the observed versus expected frequencies of all the haplotypes are considered together. The individual haplotypes were tested for association by grouping all others together and applying the χ^2-test with 1 df. P-values were calculated on the basis of 10 000 replications. All P-values reported are two tailed. Statistical significance was defined at $P < 0.05$.