toms Scale scores ($r = .29$, $p = .004$), and the Calgary Depression Scale for Schizophrenia scores ($r = .27$, $p = .006$). In addition, the Brief Psychiatric Rating Scale Negative Symptoms scores were significantly correlated with Dose of Neuroleptics ($r = .20$, $p = .05$), the Drug-induced Extrapyramidal Symptoms Scale scores ($r = .30$, $p = .002$), and the Calgary Depression Scale for Schizophrenia scores ($r = .27$, $p = .007$). Moreover, the Calgary Depression Scale for Schizophrenia scores correlated with the Drug-induced Extrapyramidal Symptoms Scale scores ($r = .31$, $p = .002$), and Number of Hospitalizations was significantly correlated with Duration of Illness ($r = .24$, $p = .02$) and Dose of Neuroleptics ($r = .43$, $p < .001$).

Table 4 shows the results of stepwise regression analyses on the Schizophrenia Quality of Life Scale and the Quality of Life Scale. There was no sign of multicollinearity for the analyses. The Psychosocial scale score was predicted independently by the Calgary Depression Scale for Schizophrenia score ($\beta = .58$, $p < .001$), the Brief Psychiatric Rating Scale Positive Symptoms score ($\beta = .42$, $p < .001$), Dose of Neuroleptics ($\beta = -.22$, $p = .007$), and the Brief Psychiatric Rating Scale Negative Symptoms score ($\beta = -.18$, $p = .03$). The Calgary Depression Scale for Schizophrenia score contributed signifi-

TABLE 4
SUMMARY OF STEPWISE REGRESSION ANALYSES ON SCHIZOPHRENIA QUALITY OF LIFE SCALE AND QUALITY OF LIFE SCALE ($N = 99$)

Dependent Variable	Independent Variable	Adjusted R^2	β
Schizophrenia Quality of Life Scale			
Psychosocial	CDSS	.48‡	.58‡
	BPRS Positive Symptoms		.42‡
	Dose of Neuroleptics		−.22†
	BPRS Negative Symptoms		−.18*
Motivation and Energy	CDSS	.23‡	.48‡
Symptoms and Side-effects	BPRS Positive Symptoms	.21‡	.37†
	CDSS		.27†
	Dose of Neuroleptics		−.20*
Quality of Life Scale			
Total	BPRS Negative Symptoms	.46‡	−.53‡
	BPRS Positive Symptoms		−.24†
Interpersonal Relations	BPRS Negative Symptoms	.36‡	−.60‡
	Duration of Illness		−.21*
Instrumental Role	BPRS Negative Symptoms	.28‡	−.33†
	BPRS Positive Symptoms		−.31†
Intrapsychic Foundations	BPRS Negative Symptoms	.53‡	−.59‡
	BPRS Positive Symptoms		−.24†
Common Objects and Activities	BPRS Negative Symptoms	.33‡	−.58‡
	Duration of Illness		−.19*

Note.—CDSS = Calgary Depression Scale for Schizophrenia; BPRS = Brief Psychiatric Rating Scale. *$p < .05$. †$p < .01$. ‡$p < .001$.

cantly to the prediction of the Motivation and Energy scale score ($\beta = .48$, $p < .001$). The Symptoms and Side-effects scale score was predicted independently by the Brief Psychiatric Rating Scale Positive Symptoms score ($\beta = .37$, $p = .001$), the Calgary Depression Scale for Schizophrenia score ($\beta = .27$, $p = .004$), and Dose of Neuroleptics ($\beta = -.20$, $p = .05$). The Quality of Life Scale total score was predicted independently by the Brief Psychiatric Rating Scale Negative Symptoms score ($\beta = -.53$, $p < .001$) and the Brief Psychiatric Rating Scale Positive Symptoms score ($\beta = -.24$, $p = .006$). The Brief Psychiatric Rating Scale Negative Symptoms score ($\beta = -.60$, $p < .001$) and Duration of Illness ($\beta = -.21$, $p = .010$) contributed independently to the prediction of the Interpersonal Relations subscale. The Instrumental Role subscale was predicted independently by the Brief Psychiatric Rating Scale Negative Symptoms score ($\beta = -.33$, $p = .001$) and the Brief Psychiatric Rating Scale Positive Symptoms score ($\beta = -.31$, $p = .002$). The Intrapsychic Foundations was also predicted by the Brief Psychiatric Rating Scale Negative Symptoms score ($\beta = -.59$, $p < .001$) and the Brief Psychiatric Rating Scale Positive Symptoms score ($\beta = -.24$, $p = .003$). The Brief Psychiatric Rating Scale Negative Symptoms score ($\beta = -.58$, $p < .001$) and Duration of Illness ($\beta = -.19$, $p = .027$) contributed independently to the prediction of the Common Objects and Activities subscale.

Discussion

In general, for the relationship between subjective and objective quality of life, the results are consistent with those of Fitzgerald, *et al.* (10). The Psychosocial scale scores of the Schizophrenia Quality of Life Scale did not significantly correlate with any subscale of the Quality of Life Scale, but did with the total score. The Symptoms and Side-effects scores of the Schizophrenia Quality of Life Scale did not significantly correlate with either the total score or any of the subscales of the Quality of Life Scale. On the other hand, the score of the Motivation and Energy scale of the Schizophrenia Quality of Life Scale was significantly correlated with the Quality of Life Scale total scores and all its subscale scores. Since motivation and activity level are generally considered to be related to deficit symptom which is a core part of the Quality of Life Scale, the results may reflect it. These results indicate that there are some significant correlations between these subjective and objective quality of life measures even using schizophrenia-specific quality of life scales. The results did not support the hypothesis. However, that correlation coefficients were not very high indicates that these two measures were not related closely.

In the current study, although there were some significant correlations among the clinical variables, stepwise regression analyses indicated that depressive and positive symptoms were significant and independent predictors

of subjective quality of life. Considering the beta coefficients of the two predictors, it is obvious that the depressive symptom was the most important predictor of subjective quality of life. Kaneda (15) studied the relationship between daily neuroleptic dose and the Schizophrenia Quality of Life Scale scores in 42 male inpatients with schizophrenia. He found that higher doses of antipsychotics were significantly correlated with higher scores on the Psychosocial or Symptoms and Side-effects scales in the Schizophrenia Quality of Life Scale. However, the current study did not support these findings. The reason for the inconsistency is not clear but may reflect differences in the samples. The study sample in Kaneda's study consisted of inpatients on a long-term unit who had been receiving high doses of antipsychotics (an average dose of haloperidol equivalent 16.0 mg/day which is equal to chlorpromazine equivalent 800.0 mg/day). Moreover, being on a long-term inpatient unit might have influenced their subjective quality of life because their Schizophrenia Quality of Life Scale scores were rather higher than those of our subjects.

In the current study, the score for negative symptoms was a significant and independent predictor of the Quality of Life Scale score, and the results support findings of previous studies (10, 25). The score for positive symptoms also predicted the Quality of Life Scale total score and some subscales. However, beta coefficients of the regression analyses indicated that the Brief Psychiatric Rating Scale Negative Symptoms score was the most important predictor of the Quality of Life Scale. Although there was a significant correlation between the Calgary Depression Scale for Schizophrenia score and the Brief Psychiatric Rating Scale Negative Symptoms score in the current study, the correlation was weak ($r = .27$). Therefore, in general, the results indicate that subjective and objective quality of life have different predictors. The results supported the hypothesis.

A limitation of this study is that it is cross-sectional so interpreting regression analyses cannot be based on causality. A longitudinal study will be needed for that.

In conclusion, the current results indicate that subjective quality of life measured by the Schizophrenia Quality of Life Scale and objective quality of life assessed by the Quality of Life Scale do not correlate closely and have different predictors. Data suggest that subjective and objective quality of life should be considered separate and complementary outcome variables. Treatment efforts should be directed towards not only effective control of positive symptoms but also toward depressive and negative symptoms to improve patients' quality of life.

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Interaction between catechol-O-methyltransferase (COMT) Val108/158Met and brain-derived neurotrophic factor (BDNF) Val66Met polymorphisms in age at onset and clinical symptoms in schizophrenia

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Summary Catechol-O-methyltransferase (COMT) gene is one of the candidate genes for schizophrenia because it codes an enzyme that participates in the metabolic inactivation of dopamine and noradrenaline and a limiting factor of dopamine metabolism in the prefrontal cortex. COMT gene lies on chromosome 22q11.2, which has been associated with schizophrenia susceptibility. A single-nucleotide polymorphism of COMT gene at position 108/158 results in an amino acid substitution from valine (val) to methionine (met), which modifies its enzymatic activity and may change the brain morphology and expressional behaviors. On the other hand, brain-derived neurotrophic factor (BDNF) plays a critical role in the development of mesolimbic dopaminergic-related systems. BDNF also contains a functional single-nucleotide polymorphism at codon 66 (Val66Met) of its prodomain and this polymorphism is responsible for schizophrenia susceptibility. In this study, we first investigated the relationship between COMT Val108/158Met polymorphism and age at onset as well as levels of clinical symptoms in 158 of chronic schizophrenia inpatients and then we investigated the gene-by-gene interaction between COMT Val108/158Met polymorphism and BDNF Val66Met polymorphism with age- and sex-matched control subjects ($n=318$). We concluded that the COMT Val108/158Met polymorphism was not related to either the onset at age or the levels of clinical symptoms after long-term antipsychotic treatment in schizophrenia.

Keywords: Schizophrenia, catechol-O-methyltransferase, brain-derived neurotrophic factor, polymorphism, brief psychiatric rating scale, age at onset

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Introduction

Catechol-O-methyltransferase (COMT) gene has been considered to be one of the candidate genes for schizophrenia because it is an important enzyme that participates in the metabolic inactivation of dopamine and norepinephrine and it lies on chromosome 22q11 which has been associated with schizophrenia susceptibility (Owen et al., 2004). COMT gene contains a functional polymorphism, a single-nucleotide polymorphism at position 108/158 that results in change from valine (val) to methionine (met) and the COMT activity with Val108/158 has one fourth lower than that with Met108/158 (Lachman et al., 1996). In contrast to the striatum, dopamine transporters in the prefrontal cortex are expressed in low abundance and the variation in COMT activity has a neurobiological effect in that region (Bertolino et al., 2004). Some genetic studies have demonstrated the possible correlation between COMT Val158/108Met gene polymorphism and schizophrenia (Shifman et al., 2002; Chen et al., 2004), although some studies have failed to find any correlation (Munafo et al., 2005; Williams et al., 2005). Recently, it is reported that COMT Val108/158Met polymorphism is linked to the morphological changes in schizophrenia (Ohnishi et al., 2006).

Brain-derived neurotrophic factor (BDNF) belongs to the neurotrophic factor family that promotes the development,

regeneration, survival and maintenance of neurons (Maisonpierre et al., 1991). BDNF has also been demonstrated to modulate neurotransmitter syntheses, metabolism and release, postsynaptic ion channel fluxes, neuronal activities and long-term potentiation (Altar et al., 1997). A single-nucleotide polymorphism that results in valine (val) to methionine (met) substituition at codon 66 (Val66Met) in the prodomain of the BDNF gene was reported and 66Met BDNF has been shown to affect intracellular trafficking and activity-dependent secretion of BDNF (Egan et al., 2001). 66Met BDNF homozygotes had smaller hippocampal volume and lower scores in the Wechsler Memory Scale compared with 66Val homozygotes in normal control subjects (Egan et al., 2003). Although small size of hippocampus is reported in schizophrenia (Callicot, 1998), the results of genetic studies on the association between BDNF Val66Met polymorphism and schizophrenia have been controversial (Chen et al., 2006; Rosa et al., 2006). Recently, we reported that schizophrenic patients with Met66 BDNF had earlier onset and this BDNF polymorphism is associated with clinical symptoms in schizophrenia (Numata et al., 2006).

In this study, we performed a case-control study with COMT Val108/158Met polymorphism, and then examined whether any association exists between this polymorphism and clinical symptoms or onset age in schizophrenic patients. We also investigated the gene-by-gene interaction between COMT Val108/158Met polymorphism and BDNF Val66Met polymorphism.

Materials and methods

We used the same DNA samples as our previous study (Numata et al., 2006) from 159 inpatients (115 male and 44 female; mean age: 53.9 ± 12.8 years, mean duration of hospitalization: 12.1 years) with schizophrenia from nine psychiatric hospitals in the neighboring area of Tokushima Prefecture in Japan (population: about 820,000). All patients were Japanese and biologically unrelated. The diagnosis of schizophrenia was made by at least two experienced psychiatrists according to DSM-IV criteria (American Psychiatric Association, 1994). Clinical symptoms and antipsychotic- induced adverse effects were evaluated when blood samples were taken by the Brief Psychiatric Rating Scale (BPRS) scores (Overall and Goham, 1962) and the Drug Induced Extra-Pyramidal Symptoms Scale (DIEPSS) (Inada et al., 2002). The age at first psychotic episode was used as age at onset (mean ± SD: 25.9 ± 8.3 years) by referring to the patient's medical records. Inter- rater reliability for the BPRS and the DIEPSS was $r = 0.81$ ($p < 0.01$). If the first degree relatives of the patient were diagnosed as schizophrenia, we considered the patient as family history plus. In our samples, 72 patients received atypical and 44 patients received typical and others received both types of antipsychotics. Age- and sex-matched controls were selected from volunteers after assessing the psychiatric problems. All control subjects were Japanese, unrelated to each other, and living in Japan. All subjects signed written informed consent to participate in this study approved by the institutional ethics committees.

Genomic DNA was extracted according to standard procedures. Genotyping of COMT Val108/158Met polymorphism was performed with taqman probe according to the manufacture's instruction with ABI 7500 (Applied Biosystems, Tokyo, Japan).

Hardy-Weinberg equilibrium was tested with HWDIAG (Rogatko et al., 2002). Frequency analysis was performed with Fisher's exact test. To evaluate associations between the genotypes and age at onset, Kaplan-Meier analyses were used for survival curves. Spearman correlation coefficients (two-tailed) were used to evaluate whether clinical symptoms of schizophrenia was correlated with Met allele dose-dependency of COMT Val108/158Met polymorphism. Group mean comparisons of the BPRS among genotypic COMT Val108/158Met polymorphism were performed with the Kruskal-Wallis statistic. Multiple linear regression was performed to explore determinants of age at onset. To determine the independent and combined effects of COMT and BDNF genotypes to the BPRS scores in schizophrenia, comparisons between groups were performed by two-way ANOVA followed by multiple comparison testing using the Tamhane correction. The criterion for significance was set at $p < 0.05$ for all of the tests. Data are presented as mean ± standard deviation.

Results

The effect of COMT Val108/158Met polymorphism in schizophrenia

COMT data were available for 158 subjects (114 males and 44 females, genotyping was failed in one subject) and 318 controls (230 males and 88 females). Genotype and allele

Table 1. *Frequency of catechol-O-methltransferase and brain-derived neurotrophic factor genotypes and alleles in patients with schizophrenia and in healthy comparison subjects*

Snp	Group	Genotype			Hardy-Weinberg equilibrium	*p*-Value	Allele		*p*-Value
		Val/Val	Val/Met	Met/Met			Val	Met	
COMT Val106/156Met									
	sch ($n = 158$) (%)	59 (37.3)	85 (53.8)	14 (8.9)	0.092	0.057	203 (64.2)	113 (35.8)	0.16
	cont ($n = 317$) (%)	151 (47.6)	134 (42.3)	32 (10.1)	0.970		436 (68.8)	198 (31.2)	
BDNF Val66Met									
	sch ($n = 159$) (%)	65 (40.9)	68 (42.8)	26 (16.3)	0.528	0.40	198 (62.3)	120 (37.7)	0.16
	cont ($n = 317$) (%)	113 (35.7)	138 (43.5)	66 (20.8)	0.950		364 (57.4)	270 (42.6)	

sch Schizophrenia; *cont* control subjects. Hardy-Weinberg equilibriums are estimated by HWDIAG. *p*-values are calculated by Fisher's exact test.

Table 2. *Genotypes of catechol-O-methl transferase Val108/158Met polymorphism and clinical symptoms of patients with chronic schizophrenia* ($n=158$)

COMT genotypes	Val/Val	Val/Met	Met/Met
Age	53.3 ± 12.4	53.7 ± 13.4	57.4 ± 10.0
Age at onset	26.3 ± 8.1	25.8 ± 8.9	25.2 ± 4.8
Duration of disease (years)	27.0 ± 12.7	27.9 ± 14.1	32.1 ± 9.1
BPRS-total	40.2 ± 10.0	40.1 ± 9.6	41.8 ± 11.1
BPRS-positive	10.5 ± 4.2	10.1 ± 3.6	11.0 ± 4.6
BPRS-negative	11.4 ± 4.0	11.0 ± 4.3	11.3 ± 3.8
DIEPS	4.6 ± 3.9	4.2 ± 4.4	4.8 ± 2.9
Daily neuroleptic dosage (mg/day)	755.4 ± 534.1	729.2 ± 530.7	594.8 ± 377.4
Duration of hospitalization (years)	14.0 ± 13.5	11.0 ± 11.2	11.4 ± 11.9
Positive first-degree family history ($n=33$)	48.5%	48.5%	3.0%

BPRS Brief Psychiatric Rating Scales; *DIEPSS* Drug Induced Extra-Pyramidal Symptoms Scale.

distributions of COMT Val108/158Met polymorphism are shown in Table 1. The genotypic distributions did not deviate from the Hardy-Weinberg equilibrium at this polymorphism in both groups. No association between schizophrenia and control subjects was found in genotype or allele frequencies. The mean onset ages were 26.3 ± 8.1 for COMT Val/Val, 25.8 ± 8.9 for COMT Val/Met and 25.2 ± 4.8 for COMT Met/Met. No significant differences were observed among genotypes (log rank statistic: 0.200, $p=0.904$). No significant sex effect was observed in the effect of the COMT polymorphism on age at onset. The mean BPRS total scores were 40.2 ± 10.0 for COMT Val/Val and 39.9 ± 9.6 for COMT Val/Met, 41.7 ± 11.1 for COMT Met/Met and were not significantly different comparing these three genotypic groups with Kruskal-Wallis comparison ($p=0.838$). No significant differences were demonstrated in the COMT genotype distributions between patients with positive and negative family history ($p=0.186$). Neither chlorpromazine-equivalent dose nor the scores of the side effect scale, DIEPSS, showed significant Spearman's rank correlation with Met allele dose-dependency of COMT polymorphism ($p=0.476$ and $p=0.689$, respectively). No significant effects of sex or duration of illness were observed in the effect of the COMT polymorphism on BPRS, chlorpromazine-equivalent dose or DIEPSS.

Interaction between COMT Val108/158Met polymorphism and BDNF Val66Met polymorphism

We have previously reported Met66 BDNF homozygotes patients showed significantly earlier age at onset compared to Val66 BDNF homozygotes by the Kaplan-Meier analyses (log rank statistic: 7.51, $p=0.023$), and that the BDNF polymorphism significantly affects clinical symptoms (Numata et al., 2006). The multiple regression analyses of age at onset were performed as dependent variables. Plausible predictors (sex, education, family history, marriage status and BDNF or COMT polymorphisms) were included in the original models. The final linear regression model included the number of BDNF 66Met alleles ($p=0.022$) and marriage status ($p<0.001$) as significant variables influencing age at onset in schizophrenia. COMT polymorphism was eliminated as a significant variable ($p<0.2$). The dose of Met66 BDNF was weakly but significantly correlated with the onset age (Spearman: $r=0.162$, $p=0.042$). On the other hand, the COMT polymorphism was not significantly correlated with the onset age ($p=0.824$).

We performed two way ANOVA analyses in clinical symptoms because we could not find linear correlation between the BDNF polymorphism and BPRS scores (Numata et al., 2006). By two-way ANOVA followed by multiple comparison testing using the Tamhane correction, BDNF Val66Met polymorphism significantly affects the BPRS in our schizophrenic in-patients samples ($p=0.040$), however, there was no significant effect seen with COMT Val108/158Met polymorphism to the BPRS ($p=0.845$). There were no significant effects of DIEPSS or the medication dose in genotypes of either gene.

Discussion

We determined whether any association exists between COMT Val108/158Met polymorphism and clinical variables of schizophrenia and investigated the interaction between COMT Val108/158Met polymorphism and BDNF Val66Met polymorphism. The genotypic frequencies of two polymorphisms of those genes in our sample were almost the same ratio as those of the precedent reports of Japanese samples (Inada et al., 2003; Kunugi et al., 2004).

COMT Val108/158Met polymorphism was not related to either the onset age or the levels of clinical symptoms that remained after long-term antipsychotic treatment in our sample. It has been reported that an association between COMT Met/Met genotype and schizophrenia patients with aggressive behavior as well as suicidal behavior (Nolan et al., 2000; Strous et al., 2003). A possible interaction between low activity COMT and poor response to conventional neuroleptics has been suggested (Illi et al., 2003). However, the lack of association between this COMT polymorphism and clinical variables of schizophre-

nia in this study is consistent with previous reports (Herken et al., 2003; Strous et al., 2006). No significant effects of age at onset or duration of illness were observed in the effect of the COMT polymorphism on the BPRS.

Since COMT knockout mice are known to have increased brain dopamine, especially in the frontal cortex and to show aberrant behavior (Gogos et al., 1998), there may be a distinct effect of the functional single nucleotide polymorphism, COMT Val108/158Met, in human behaviors and diseases. Several studies have revealed that subjects with COMT Met/Met homozygotes performed better than COMT Val/Val homozygotes on executing the Wisconsin Card Sorting Test (WCST), a test associated with prefrontal cortical function (Egan et al., 2001; Malhotra et al., 2002). Ohnishi et al found that this COMT polymorphism is associated with morphological changes in schizophrenia, particularly in the limbic and paralimbic systems (Ohnishi et al., 2006). More extensive studies on the association between the COMT polymorphism and clinical variables are necessary.

Schizophrenia is a complex psychiatric disorder with multiple factors including genetic inheritances. We hypothesized that gene-by-gene interaction might contribute to the different effects of COMT Val108/158Met polymorphism on clinical variables of schizophrenia. We have previously found that the BDNF gene Val66Met polymorphism is related to the onset age of schizophrenia and also influences to the levels of clinical symptoms that are refractory to long-term ordinary antipsychotic treatment in the same sample (Numata et al., 2006). Gourion et al. reported that interaction between BDNF Val66Met and dopamine D3 receptor Ser9Gly polymorphisms was significantly associated with an earlier emergence of psychosis by three years (Gourion et al., 2005). So we investigated the gene by gene interaction between COMT Val108/158Met polymorphism and BDNF Val66Met polymorphism, but the COMT Val108/158Met × BDNF Val66Met genotype interactions were not detected in this study. However, The BDNF Val66Met polymorphism still indicates a weak but significant effect to onset age and the BPRS even after adjusting for the COMT genetic effect. Kaufman et al. reported that children with one or two of Met66 BDNF alleles are vulnerable to environmental stress in depression (Kaufman et al., 2006). We suggest that the schizophrenic patients with Met66 BDNF might also show vulnerability to environmental stress and suffer the disease earlier.

Our study has several limitations. First, the BPRS is a cross sectional rating scale but not a life time scale, although our patients showed little fluctuation in their symptoms at the time of the interview under long-term antipsychotic treatment. Second, all the patients were long-term inpatients and might not represent schizophrenic patients in general. Third, the sample size is relatively small. Larger studies will be needed to confirm these results.

In summary, our finding suggests that, unlike BDNF Val66Met polymorphism, COMT Val108/158Met polymorphism is not related to the onset age of schizophrenia and does not influence to the levels of clinical symptoms that are refractory to long-term ordinary antipsychotic treatment at least in the Japanese population.

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Altered HDAC5 and CREB mRNA expressions in the peripheral leukocytes of major depression

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Abstract

Background: Gene expressions of the peripheral leukocytes in depressive patients might reflect the systemic dysfunction of major depression. We determined mRNA expression levels of Histone deacetylase 5 (HDAC5) gene and cyclic AMP response element-binding protein 1 (CREB) gene in the leukocyte of depressive patients. HDAC5 and CREB are reported to be important targets of antidepressants, the latter being located in the downstream of the former in lymphocyte calcium signaling.
Methods: 25 patients with major depression and 25 age- and sex-matched healthy controls were included in this study. Twenty patients were able to be followed up until the 8 week-treatment. The mRNA levels were determined by a quantitative RT-PCR method.
Result: Levels of *HDAC5* and *CREB* mRNA were significantly higher in drug-free depressive patients than those of controls and the higher mRNA levels decreased to control levels after 8-week paroxetine treatment. There were positive correlation between levels of *HDAC5* and *CREB*.
Conclusion: Our results suggest the alteration of HDAC5 and CREB gene expression in the systemic pathophysiology of major depression.

Keywords: Calcium signaling; CREB; HDAC5; Leukocyte; Major depression; mRNA expression

1. Introduction

Several studies including our own have shown the altered mRNA expressions in the peripheral leukocytes of patients with major depression (Rocca et al., 2002; Iga et al., 2005, 2006). For example, we reported that the level of serotonin transporter mRNA in leukocytes was higher in depressive patients than that of control subjects, and normalized after antidepressant treatment (Iga et al., 2005). Not only neurochemical transmitter systems, such as serotonergic and noradrenergic, but also hormones, cytokines and even structural molecules in the whole body may take part in the occurrence of depressive states (Raison and Miller, 2003; Henn and Vollmayr, 2005). Thus, gene expressions of the peripheral leukocytes in depressive patients may reflect the systemic dysfunction and the response to antidepressants.

Recent progress in molecular pharmacology of antidepressants highlighted the importance of chromatin remodeling in controlling gene expression by regulating the histone acetylation and methylation (Berton and Nestler, 2006; Newton and Duman, 2006). Histone deacetylase 5 (HDAC5) is one of the interesting targets of antidepressants, because it was reported that the down-regulation of HDAC5 by chronic antidepressant treatment was critical for its therapeutic efficacy in the animal model of depression (Tsankova et al., 2006). HDAC5 is also known to be involved in calcium/calmodulin-dependent protein kinase signaling and control of cellular differentiation (McKinsey et al., 2000).

Cyclic AMP response element-binding protein 1 (CREB), which is one of transcriptional targets of the calcium signaling, has been reported to be a molecular marker for the response to antidepressants in neurons (Blendy, 2006). Interestingly, alteration of calcium signaling in the peripheral leukocytes has been reported in major depression (Bohus et al., 1996; Iga et al., 2006; Vollmayr et al., 1995). For example, we reported that the

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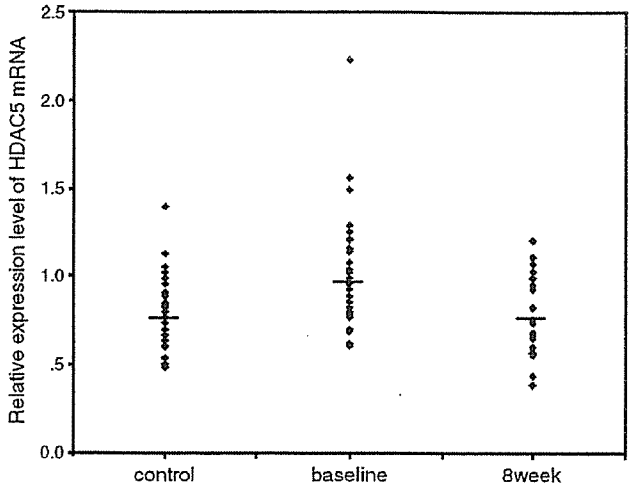

Fig. 1. *HDAC5* mRNA expression levels in the peripheral leukocytes. Bars indicate the mean of the values of each subject (control, age- and sex-matched controls, N=25; baseline, patients before treatment, N=25; 8-week, Patients after 8-week paroxetine treatment, N=20). The *HDAC5* mRNA levels were significantly higher in patients (control; 0.92±0.21, baseline; 0.77±0.16: Student t test P=0.008) and were significantly decreased at 8-week compared with those at baseline (N=20, baseline; 0.92±0.20, 8-week; 0.76±0.23: paired t test P=0.031).

PDLIM5 gene, which is involved in calcium signaling in neurons, showed lower expression in the leukocytes of depressive patients and the expression was increased up to healthy control levels after paroxetine treatment. We suggest that the calcium signaling in the leukocytes may be a useful biological marker of major depression (Iga et al., 2006). Since CREB is downstream of HDAC5 in lymphocyte calcium signaling and both are targets of antidepressants in neurons (Gallo et al., 2006; West et al., 2002), we hypothesize that the levels of *HDAC5* and *CREB* mRNA may be altered in the leukocytes of major depression.

2. Materials and methods

The subjects were 25 patients diagnosed as Major Depressive Disorder according to DSM-IV (APA, 1994) (8 males, 17 females and mean age 41.1±13.1) and 25 age- and sex-matched controls (mean age 41.7±13.3). Before study participation, all subjects signed an informed consent form approved by the Ethical Committee of University of Tokushima School of Medicine. All patients 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. Eighteen patients were in the first and other seven were in the recurrent depressive episode. All patients did not receive any antidepressants for the current episode before blood sampling. We were able to follow up twenty patients 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, patients were rated with Structured Interview Guide for the 17-item Hamilton Depression Rating Scale (SIGH-D 17, Williams, 1988) before blood collection. Peripheral blood was also collected from 25 sex- and age-matched volunteers who were in good physical health with a history of neither psychiatric nor serious somatic disease and were not taking any medication. Probands who had first-degree relatives with psychiatric disorders were excluded from the control subjects.

2.1. 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 which the separation was performed on a Phenomenex C18 column (4.6×250 mm).

2.2. Quantitative real-time PCR

Total RNA was extracted from the peripheral leukocytes of whole blood samples using the PAXgene 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 (Assay ID: Hs00608366_m1 for HDAC5, Hs01081733_m1 for CREB, Applied Biosystems, CA, USA). We used two control genes (Glyceraldehyde-

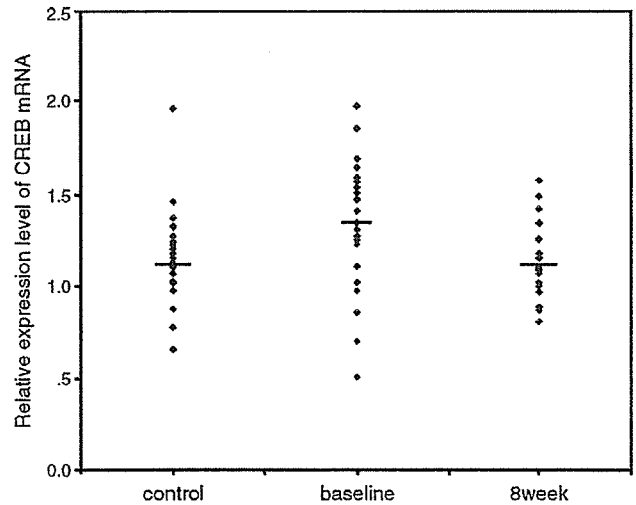

Fig. 2. *CREB* mRNA expression levels in the peripheral leukocytes. Bars indicate the mean of the values of each subject (control, age- and sex-matched controls, N=25; baseline, patients before treatment, N=25; 8-week, Patients after 8-week paroxetine treatment, N=20). The *CREB* mRNA levels were significantly higher in patients. (control; 1.17±0.25, baseline; 1.35±0.35: Student t test, P=0.034) and were significantly decreased at 8-week compared with those at baseline (N=20, baseline; 1.38±0.35, 8-week; 1.15±0.21, paired t test P=0.021).

Table 1
Characteristics of patients

Age/sex[a]	Episode	Age of onset	Hereditary load
25/M (25/M)	Single	25	–
26/F (27/F)	Single	25	–
26/F (29/F)	Single	26	–
26/F (29/F)	Single	25	+
30/F (33/F)	Single	30	–
30/M (30/M)	Single	30	–
32/M (32/M)	Single	32	–
33/M (31/M)	Single	32	–
33/F (36/F)	Single	32	–
37/M (37/M)	Single	37	–
41/F (44/F)	Single	41	–
46/F (48/F)	Single	46	–
49/M (48/M)	Single	49	–
52/F (53/F)	Single	52	–
56/M (61/M)	Single	56	–
60/F (58/F)	Single	60	–
63/M (64/M)	Single	63	–
66/F (68/F)	Single	66	+
28/F (28/F)	Recurrent	16	+
30/F (29/F)	Recurrent	28	–
38/F (33/F)	Recurrent	37	+
43/F (40/F)	Recurrent	42	–
45/F (45/F)	Recurrent	43	–
56/F (58/F)	Recurrent	54	–
57/F (57/F)	Recurrent	53	–

[a]Data of the respective matched control subject presented in parentheses.

3-phosphate dehydrogenase: GAPDH and Hypoxanthine guanine phosphoribosyltransferase 1: HPRT) for normalization of possible fluctuations in quantitative values of the target transcripts (Applied Biosystems). Measurements of each gene expression with Delta CT method were conducted in triplicate.

2.3. 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 Mann–Whitney U test. The changes before and after treatment were calculated with the Wilcoxon rank sum test. Spearman correlation coefficients were used to evaluate the correlations. All significance levels were two-sided. The criterion for significance was set at $P<0.05$ for all tests. Data are presented as mean±standard deviation.

3. Result

The relative amount of *HDAC5* mRNA in the peripheral leukocytes was standardized with *GAPDH* mRNA. We also used HPRT mRNA as a standard but obtained almost the same results (data not shown).

The *HDAC5* mRNA levels are shown in Fig. 1. The *HDAC5* mRNA levels were significantly higher in patients (Patients; 0.92±0.21, Controls; 0.77±0.16: Mann–Whitney U test, $P=0.020$) and were significantly decreased at 8-week compared with those at baseline ($N=20$, baseline; 0.92±0.20, 8-week; 0.76±0.23: Wilcoxon rank sum test, $P=0.033$).

The *CREB* mRNA levels are shown in Fig. 2. The *CREB* mRNA levels were significantly higher in patients (Controls; 1.17±0.25, Patients; 1.35±0.35: Mann–Whitney U test, $P=0.009$) and were significantly decreased at 8-week compared with those at baseline ($N=20$, baseline; 1.38±0.35, 8-week; 1.15±0.21, Wilcoxon rank sum test, $P=0.028$).

The characteristics of patients are shown in Table 1. No clinical variables (ages, sexes, number of episodes, age of onset, hereditary load or HAM-D scores (21.2±7.3)) showed significant correlations with the *HDAC5* or *CREB* levels before treatment. Interestingly, positive correlation of the mRNA expression was observed between *HDAC5* and *CREB* before treatment ($P=0.007$ $r=0.529$; Spearman correlation test, Fig. 3).

Mean paroxetine doses and concentration at 8 week-treatment were 30.5±8.9 mg/day and 77.3±58.9 ng/ml, respectively. HAM-D scores were significantly improved after

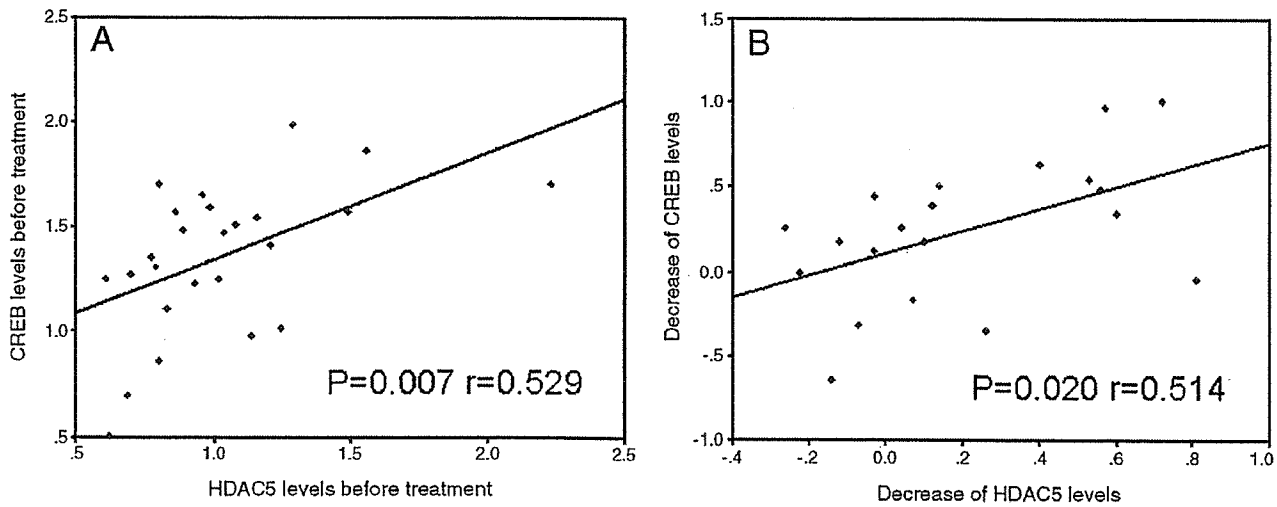

Fig. 3. Correlations between the levels of *HDAC5* mRNA and *CREB* mRNA before treatment (A: $N=25$) and decrease of *HDAC5* and *CREB* during the treatment (B: $N=20$) are shown. Each point represents an individual and the solid lines represent the regression of the correlations. Both axes are expressed as relative expression levels of target genes.

8-week paroxetine treatment (8.4±6.6, $P<0.001$). Neither paroxetine concentrations nor the changes of HAM-D scores showed significant correlations with the changes of the *HDAC5* or *CREB* levels. Positive correlation was also observed between the changes of *HDAC5* and the changes of *CREB* levels during 8 week-treatment $P=0.020$ $r=0.514$; Spearman correlation test, Fig. 3).

4. Discussion

The importance of specific chromatin remodeling is supported by recent articles demonstrating that modification of histone acetylation, by administration of inhibitors or viral-mediated over expression of specific HDACs, alters behavior in models of depression (Cassel et al., 2006; Tsankova et al., 2006). One particularly interesting target is HDAC5 because *HDAC5* mRNA is decreased by antidepressant treatment and when over-expressed was found to block the behavioral effects of antidepressants (Tsankova et al., 2006). It is generally believed that hyperacetylation of histones catalyzed by histone acetyl-transferases (HATs) facilitates gene transcription and the action of HATs is opposed by HDACs. HDACs and HATs are controlled mainly at the level of their recruitment to target promoters, but some evidences suggest that at least *CREB*-binding protein, a type of HAT, may be regulated directly through calcium signaling (West et al., 2002). Because both HDAC5 and CREB are also involved in the lymphocyte calcium signaling and may be associated with histone modification, our finding that their mRNA levels are up-regulated in the leukocytes of depressive patients before treatment may reflect impairments of calcium signaling which lead to the abnormal chromatin remodeling. The higher expression of *CREB* mRNA in antidepressant-free depressive patients is not consistent with a previous study showing no significant difference of CREB mRNA levels in the peripheral lymphocytes of depressive patients (Lai et al., 2003). Some differences in the experimental procedures such as extraction kit (TriZOL vs. Pax gene) or housekeeping gene (beta actin vs. GAPDH and HPRT) may explain this discrepancy. The changes of CREB levels in postmortem brain tissue of major depression are also controversial. For example, Odagaki et al. (2001) reported that the increases in CREB were specifically observed in prefrontal cortex of antidepressant drug-free subjects but not in the antidepressant-treated subjects, while Yamada et al. (2003) reported that CREB was significantly decreased in orbitofrontal cortex of antidepressant-free depressive patients compared to controls.

Down-regulation of *HDAC5* mRNA after paroxetine treatment is consistent with a study showing down-regulation of *Hdac5* mRNA in the hippocampal neurons after chronic antidepressant administration (Tsankova et al., 2006). Our result suggests that the chromatin remodeling induced by the down-regulation of *HDAC5* mRNA is an important mechanism controlling long-term adaptive changes of the antidepressant in not only mice but human tissues. Down-regulation of *CREB* mRNA after paroxetine treatment is also consistent with the Lai's study showing down-regulation of *CREB* mRNA in the lymphocytes of depressive patients after chronic antidepressant administration (Lai et al., 2003). Although many animal studies demonstrate that chronic antidepressants or electroconvulsive seizures increase CREB in hippocampus (Nibuya et al., 1996; Jeon et al., 1997; Thome et al., 2000), it has been reported that inhibition of CREB by viral-mediated over expression of a dominant-negative mutant CREB that blocks CREB function in the nucleus accumbens results in antidepressant-like responses (Newton et al., 2002; Pliakas et al., 2001).

There were positive correlations between mRNA levels of *HDAC5* and *CREB* before treatment as well as between their changes during 8 week-treatment. Because CREB is down-stream of HDAC5 in lymphocyte calcium signaling (Gallo et al., 2006), it is not surprising that the mRNA expression of CREB and HDAC5 showed the same changes to the same treatment. Because there was no correlation between mRNA levels of HDAC5 and CREB in controls ($P=0.679$ $r=0.087$; Spearman correlation test), the positive correlations between mRNA levels of *HDAC5* and *CREB* may be associated with the pathophysiology and treatment of major depression.

Both HDAC5 and CREB may be important factors in the pathophysiology of bipolar disorder (BPD), because valproate, a therapeutic agent for BPD, is known to regulate gene expression by acting as a histone deacetylase inhibitor and CREB has been reported to be involved in the neurotrophic hypothesis of bipolar disorder (reviewed in Zarate et al., 2006). Although it is difficult to point out the similarities and differences between the findings in MDD and BPD, we can make some discussions. Valproate is known to inhibit non-selectively all subtypes of HDACs (Gottlicher, 2004), while antidepressant imipramine selectively inhibit HDAC5 and promote BDNF expression in hippocampus (Tsankova et al., 2006). The different selectivity may be associated with the different therapeutic properties between antidepressant and valproate. Antidepressant treatment may increase CREB levels in temporal cortex of subjects with major depressive disorder, but anticonvulsant mood stabilizing drugs may have opposite effects on cortical CREB levels in patients with bipolar disorder (Stewart et al., 2001).

In conclusion, increased levels of *HDAC5* and *CREB* mRNA in the leukocytes may be a useful biological marker of major depression. Altered expression of these genes may be associated with the systemic pathophysiology of depression and normalization to control levels after treatment may be related to the antidepressant effect. Further clinical and experimental studies are necessary to confirm and extend the present results.

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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.

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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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-nitro-benzo-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.

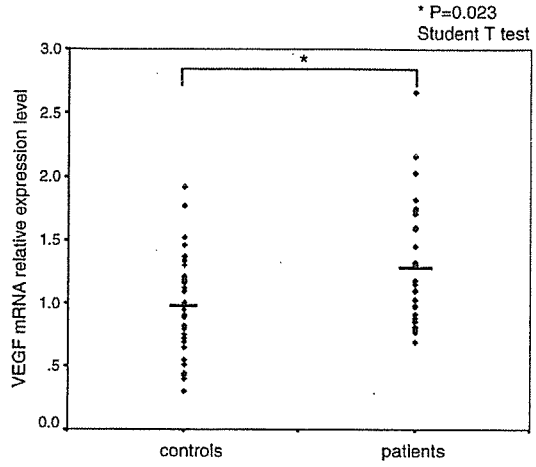

Fig. 1. VEGF mRNA expression levels before treatment. Bars indicate the mean of the values of each subject (control, age- and sex-matched controls, $N=32$; baseline, patients before treatment, $N=32$). The VEGF mRNA levels were significantly higher in patients (control; 0.92±0.21, baseline; 1.23±0.5: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

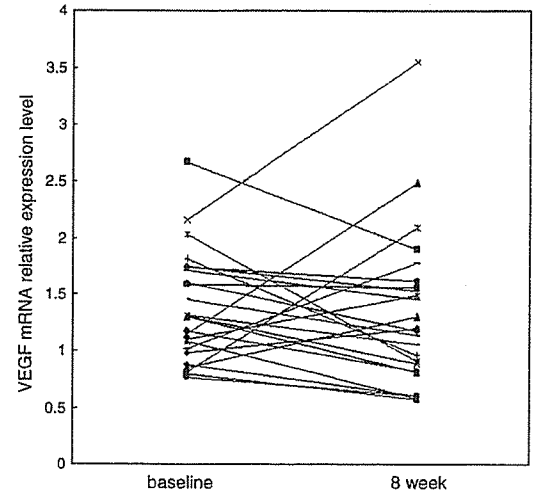

Fig. 2. VEGF mRNA expression levels during the treatment. Patients before treatment ($N=24$) and patients after 8-week paroxetine treatment ($N=24$) are connected by the solid lines. The VEGF mRNA levels did not show a significant change during the treatment (baseline; 1.37±0.49, 8-week; 1.34±0.69, paired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

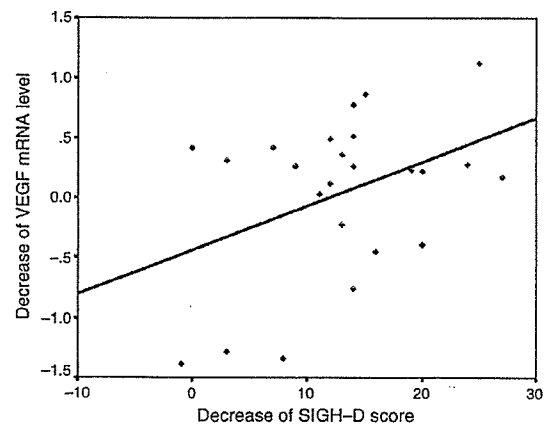

Fig. 3. Significant correlations between the decrease of VEGF mRNA and improvement of SIGH-D score (Pearson correlation $r=0.41$ $P=0.049$, $N=24$). Each point represents an individual and the solid lines represent the regression of the correlations.

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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