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Reduced expression of glyoxalase-1 mRNA in mood disorder patients

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ABSTRACT

Glyoxalase-1 (Glo1) is an antioxidant enzyme which detoxifies α-ketoaldehydes to prevent the accumulation of pro-oxidant compounds, such as methylglyoxal, in all cell types. Glo1 has been suggested to be involved in anxiety disorders, autism, and Alzheimer's disease. Mood disorders have a high rate of comorbidity with anxiety disorders although, to date, little is known of the involvement of Glo1 in the pathophysiology of these conditions. In the present study, we examined the expression levels of Glo1 mRNA in peripheral white blood cells of mood disorder patients to understand the role of Glo1 in mood disorders. Quantitative real-time polymerase chain reaction experiments revealed that reduced expression of Glo1 mRNA was observed in major depressive and bipolar disorder patients in a current depressive state, as compared with healthy control subjects. In contrast, the expression of Glo1 mRNA in major depressive and bipolar patients, in a remissive state, showed no significant alteration when compared with healthy control subjects. These results suggest that the aberrant expression of Glo1 might be involved in the pathophysiology of mood disorders.

A growing body of evidence has implicated a role of chronic or moderate oxidative stress in the pathogenesis of anxiety in humans [2]. Previous clinical investigations have reported an imbalance of antioxidant enzyme activities in patients with social phobia and obsessive-compulsive disorder [17]. Glyoxalase-1 (Glo1) is an antioxidant enzyme that, together with the cofactor glutathione, is involved in the detoxification of α-ketoaldehydes, thereby preventing the accumulation of pro-oxidant compounds such as methylglyoxal [27,28]. The association between altered Glo1 expression levels and anxiety disorders in mice supports the hypothesis that Glo1 is involved in the pathogenesis of these conditions [11,15].

The manifestation of anxiety in a number of psychiatric disorders such as generalized anxiety disorder, depressive disorder, panic disorder, phobia, obsessive-compulsive disorder and post-traumatic stress disorder [6] highlights the importance of gaining a better understanding of common biomarkers for these disorders. The significant association between anxiety and depression in behavioral studies [15] resembles the clinical situation of a high comorbidity between anxiety disorders and major depressive disorder [18]. Although Glo1 has been reported to be associated with anxiety [22], little is known about the involvement of Glo1 in the pathophysiology of mood disorders. To investigate the role of Glo1 in the pathophysiology of mood disorders, we examined the expression levels of Glo1 mRNA in the peripheral white blood cells of major depressive and bipolar disorder patients in a depressive, as well as a remissive, state.

Major depressive and bipolar disorder patients were diagnosed according to the criteria in the Diagnostic and Statistical Manual of Mental Disorders, fourth edition (DSM-IV) [1]. These included both outpatients and inpatients of the Division of Neuropsychiatry of the Yamaguchi University Hospital. The extent of the depressive state was assessed by a 21-item "Hamilton depression rating scale" (HDRS). Subjects were classified as being under a current depressive state when they showed a score of more than 18 on HDRS and met the DSM-IV criteria for a major depressive episode. Subjects were classified as being in remission when they showed a score of less than six on HDRS and did not show any symptoms of a major depressive episode in the DSM-IV criteria for more than 2 months. Individuals were excluded from the present study if they had abnormal physical examinations or abnormal results for routine medical laboratory tests such as a complete blood count and renal, liver or thyroid function. Female subjects who were pregnant or took oral contraceptives were also excluded. All healthy control subjects were screened to exclude significant current or past medical or neurological illnesses, significant alcohol or drug abuse and past or current axis I psychiatric illnesses. This protocol was approved by the Institutional Review Board of Yamaguchi University Hospital. Informed written consent was obtained for all subjects.

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Blood sample preparation, total RNA isolation and cDNA synthesis were performed as previously described [19]. In brief, blood was obtained by vein puncture between 10:00 a.m. and 11:00 a.m. and total RNA was isolated using the QIAamp RNA blood mini kit (Qiagen, Chatsworth, CA) according to the manufacturer's manual. One microgram of total RNA was used for cDNA synthesis using random hexamer primers and omniscript reverse transcriptase (Qiagen). The cDNA was stored at −80 °C until use. Quantitative real-time polymerase chain reaction (PCR) was performed in an Applied Biosystems 7300 fast real-time PCR system with SYBR green PCR master mix (Applied Biosystems, Foster City, CA), as previously reported [19]. PCR conditions were 15 min at 95 °C, 45 cycles of 15 s at 95 °C and 30 s at 60 °C. Amplification of the single PCR product was confirmed by monitoring the dissociation curve and electrophoresis on 1% agarose gels stained with ethidium bromide. The expression level of GAPDH mRNA was used for normalization and the expression value was normalized by dividing the mean of the value for control subjects. All measurements were performed in duplicate and two-independent experiments were conducted. The following PCR primers were used: Glo1 forward, 5′-CGAGGATTCGGTCATATTGG-3′; Glo1 reverse, 5′-CCAGGCCTTTC-ATTTTACCA-3′; GAPDH forward, 5′-CAGCCTCAAGATCATCAGCA-3′; GAPDH reverse, 5′-TGTGGTCATGAGTCCTTCCA-3′. A subgroup of subjects in a current depressive state underwent the dexamethasone (Dex)/corticotropin-releasing hormone (CRH) test as previously reported [19]. All data are expressed as means ± standard error of the mean (SEM). Statistical analysis was performed with commercial software (SPSS version 16.0; Chicago, IL). Multivariable analysis was conducted using Glo1 mRNA level as a dependent variable and with age, gender, state (depressive and remissive states) and type of drugs used (antidepressants and mood stabilizers) as independent variables. Gender distribution was analyzed by the χ^2-test. The data of Glo1 mRNA levels were subjected to a factorial analysis of variance (ANOVA) followed by *post hoc* comparison (Dunnett test). The Spearman rank correlation was calculated to assess the correlation between data. Two group comparisons, such as suppressors and non-suppressors of the Dex/CRH test on Glo1 mRNA expression, were performed using the Student's *t*-test. In all cases, *p*-values were two-tailed, and comparisons were considered to be statistically significant for $p < 0.05$.

Table 1 shows the demographic and clinical characteristics of the subjects used in this study. The mean ages were not significantly different among major depressive disorder patients, bipolar disorder patients and healthy control subjects ($F_{(2, 104)} = 1.84$, $p = 0.16$). Regarding the gender distribution, bipolar disorder patients showed a significantly larger ratio of females to males

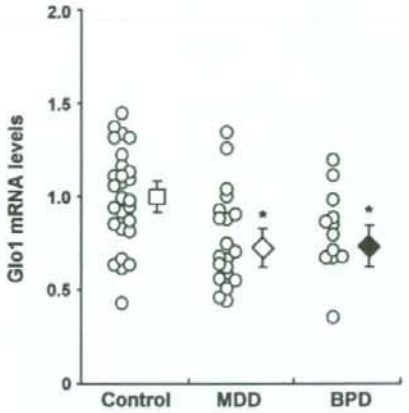

Fig. 1. Expression levels of Glo1 mRNA for mood disorder patients in a current depressive state. Quantitative real-time PCR experiments revealed reduced expression levels of Glo1 mRNA (open circles) for major depressive disorder patients in a current depressive state (MDD, $n = 20$) and bipolar disorder patients (BPD, $n = 13$), as compared to normal control subjects ($n = 28$). Data is represented as means ± S.E.M. (control, open square; MDD, open diamond; BPD, closed diamond). Asterisk represents statistically significant difference at $p < 0.05$.

($\chi^2 = 11.77$, $p = 0.001$). Multivariable analyses demonstrated that the variable "state (depressive and remissive states)" was solely and significantly associated with the expression level of Glo1 mRNA ($p = 0.004$), when analyzed together with the control variables: age, gender, and type of drugs used (antidepressants and mood stabilizers). Quantitative real-time PCR experiments revealed that reduced expression of Glo1 mRNA was observed in major depressive disorder patients ($F_{(2, 58)} = 5.70$, $p < 0.01$) and bipolar disorder patients in a current depressive state ($F_{(2, 58)} = 5.70$, $p < 0.05$), compared with healthy control subjects (Fig. 1). In a remissive state, by contrast, there was no significant difference in the expression levels of Glo1 mRNA in major depressive disorder patients ($F_{(2, 98)} = 0.19$, $p = 0.82$) or bipolar disorder patients ($F_{(2, 98)} = 0.19$, $p = 1.00$), compared with healthy control subjects (Fig. 2). There was a significant correlation between Glo1 mRNA levels and HDRS scores in major depressive disorder patients ($r = -0.358$, $p = 0.005$) (Fig. 3), but not in bipolar disorder patients ($r = -0.198$, $p = 0.187$).

Dysfunction of the hypothalamic–pituitary–adrenal (HPA) system is the most characteristic biological alteration found in the majority of depressed patients. Accumulating evidence suggests

Table 1
Demographic and clinical characteristics of subjects

	Controls	Patients			
		MDD		BPD	
		Depressive	Remissive	Depressive	Remissive
Number of subjects	28	20	40	13	33
Mean age (years)	50.0 ± 1.8	52.3 ± 3.5	57.2 ± 2.2	55.5 ± 3.5	52.7 ± 2.6
Gender (male/female)	15/13	10/10	15/25	2/11	7/26
HDRS		25.9 ± 1.9	3.3 ± 0.2	24.6 ± 1.0	2.8 ± 0.2
Medication					
No medication	28	3	4	1	0
SSRI/SNRI	0	10	38	9	9
TCA/other antidepressants	0	23	28	6	14
Li	0	0	2	4	17
VPA	0	0	0	7	15
CBZ	0	0	0	2	8

MDD, major depressive disorder; BPD, bipolar disorder; HDRS, Hamilton depression rating scale; SSRI, selective serotonin reuptake inhibitor; SNRI, serotonin–noradrenaline reuptake inhibitor; TCA, tricyclic antidepressant; Li, lithium; VPA, valproic acid; CBZ, carbamazepine.

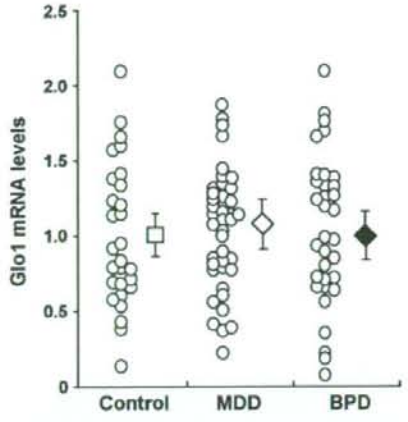

Fig. 2. Expression levels of Glo1 mRNA for mood disorder patients in a remissive state. Quantitative real-time PCR experiments revealed that expression of Glo1 mRNA (open circles) for major depressive disorder patients in a remissive state (MDD, $n = 40$) and bipolar disorder patients (BPD, $n = 33$) were not significantly different to that of normal control subjects ($n = 28$). Data is represented as means ± S.E.M. (control, open square; MDD, open diamond; BPD, closed diamond).

that the combined Dex/CRH test is highly sensitive and is able to detect HPA system abnormalities [10]. ACTH and cortisol responses to this test are exaggerated in depressed patients [7,8]. To examine the association between Glo1 mRNA levels and HPA axis activity, the mRNA levels for Glo1 of mood disorder patients in a current depressive state were compared between suppressors ($n = 11$; 8 major depressive disorder patients and three bipolar disorder patients) and non-suppressors ($n = 15$; 8 major depressive disorder patients and seven bipolar disorder patients) of the Dex/CRH test. There was no significant difference in the expression levels of Glo1 mRNA between suppressors and non-suppressors ($F_{(1,24)} = 3.68$, $p = 0.67$). In addition, there was no significant correlation between Glo1 mRNA levels and the plasma cortisol concentration in healthy control subjects ($r = -0.09$, $p = 0.72$), major depressive disorder patients ($r = 0.42$, $p = 0.27$) or bipolar disorder patients ($r = -0.50$, $p = 0.39$).

Previous reports have suggested the involvement of Glo1 in neuropsychiatric disorders, including anxiety disorders and autism. A significant association of the Glo1 Ala111Glu polymorphism has been observed in a subgroup of patients with panic disorder without agoraphobia [22] and patients with autism [12]. Reduced Glo1 enzyme activity has also been observed in the brains of patients

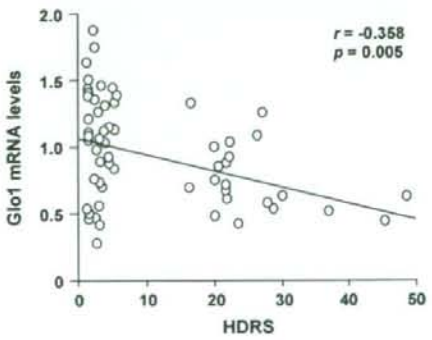

Fig. 3. Significant inverse correlation between HDRS scores and Glo1 mRNA levels was found in the major depressive disorder patients ($n = 60$). HDRS, Hamilton depression rating scale.

with autism [12]. Moreover, a possible association between Glo1 and mood disorders has been found in a linkage study of families with mood disorders [26]. There is a wealth of data demonstrating the comorbidity of mood disorders with anxiety disorders [3,31,24], including panic disorder [5,13,25]. Genetic data with regard to panic disorder and major depressive disorder have been inconsistent, although there is some evidence for a shared diathesis for anxiety and depression [29]. These data suggest an important role for Glo1 in the pathophysiology of many neuropsychiatric disorders, especially with regard to the anxiety symptoms of these conditions.

Krömer et al. [15] have reported an association between reduced Glo1 expression and high anxiety-like behaviors in mice. Importantly, the reduced expression of Glo1 was observed not only in the amygdala, but also in peripheral red blood cells [15], suggesting that the expression levels of Glo1 in the brain is well correlated with that in peripheral blood cells. These data and our present study raise the possibility that the expression levels of Glo1 in mood disorder patients may be reduced in multiple systems. However, a recent study has shown that local overexpression of Glo1 in various brain regions, e.g. cingulate cortex, resulted in increased anxiety-like behavior [11]. This finding is discordant with that of Krömer et al. [15] and thus, it is still unclear how Glo1 is involved in the pathophysiology of anxiety and depression.

A previous report has shown that the number of Glo1 immunopositive neurons and astroglia increase up to, approximately, 55 years of age and decrease progressively thereafter in humans [16]. Glo1 mRNA levels also showed a biphasic course similar to those observed with protein determination [16], suggesting that the expression of Glo1 is primarily regulated at the transcriptional level. The promoter region of the human Glo1 gene contains several consensus sequences for known transcriptional regulatory elements, including: insulin responsive element, metal responsive element and glucocorticoid responsive element [23]. The existence of the glucocorticoid responsive element in the human Glo1 promoter is particularly interesting, because the glucocorticoid receptor (GR) has been shown to be associated with mood disorders and in the adaptation to stress [4,9,20]. Reduced expression of GRα has been observed in the cerebral cortex, hippocampus and amygdala in mood disorder patients [30,14,21]. In addition, we have previously reported that the expression of GRα mRNA is also reduced in the peripheral white blood cells of mood disorder patients [19]. This raises the possibility that dysfunction of GR plays a causal role in the aberrant Glo1 expression observed in mood disorder patients.

Considering our results from multivariable analysis and the significant correlation between Glo1 mRNA levels and HDRS scores in major depressive disorders, it could be interpreted that the reduced expression of Glo1 mRNA is "state-dependent" at least in major depressive disorders. However, our study has the limitation that all the patients were on medication; therefore, we cannot exclude completely the influence of medication on the expression levels of Glo1 mRNA. To our knowledge, however, there is no evidence showing altered levels of Glo1 expression by treatment with antidepressants or mood stabilizers *in vitro* or *in vivo*. Further study conducted in medication-free subjects is needed to elucidate this issue.

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Reduced Glucocorticoid Receptor α Expression in Mood Disorder Patients and First-Degree Relatives

Toshio Matsubara, Hiromasa Funato, Ayumi Kobayashi, Masaaki Nobumoto, and Yoshifumi Watanabe

Background: *Individuals with mood disorders exhibit altered function of the hypothalamic-pituitary-adrenal (HPA) axis in response to stress. The glucocorticoid receptor (GR) plays an important role in the negative feedback regulation of the HPA axis. There are two protein isoforms of GR, GRα and GRβ, which have distinct biological activity. It has not been examined whether GRα messenger RNA (mRNA) and GRβ mRNA expressions are altered in peripheral blood cells of mood disorder patients.*

Methods: *Using quantitative reverse transcription polymerase chain reaction (RT-PCR), GRα mRNA and GRβ mRNA were measured in peripheral blood cells of major depressive disorder patients (depressive n = 18; remissive n = 38), bipolar disorder patients (depressive n = 13; remissive n = 35), normal control subjects (n = 31), and first-degree relatives of major depressive (n = 17) and bipolar (n = 15) disorder patients.*

Results: *Reduced expression of GRα mRNA was shown in both bipolar and major depressive disorder patients in a current depressive state as well as in remission. First-degree relatives of bipolar disorder patients also showed GRα mRNA reduction. Altered GRβ mRNA expression was not found in mood disorder patients.*

Conclusions: *Our results suggest that reduced GRα mRNA expression might be trait-dependent and associated with the pathophysiology of mood disorders.*

Key Words: Glucocorticoid receptor isoforms, α, β, mood disorder, first-degree relatives of mood disorders, HPA axis, trait-marker, DEX/CRH test

Individuals with mood disorders often exhibit hyperactivity of the hypothalamic-pituitary-adrenal (HPA) axis, such as increased concentration of plasma cortisol and blunted suppression to dexamethasone as measured by the dexamethasone suppression test and dexamethasone/corticotropin-releasing hormone (DEX/CRH) test (Arborelius et al 1999; Holsboer 2001; Nestler et al 2002; De Kloet 2003). Although the molecular mechanism of aberrant regulation of the HPA axis in mood disorder patients remains unclear, one candidate is the dysfunction of the glucocorticoid receptor (GR), which plays an important role in the negative feedback of the HPA axis and adaptation to stress (Holsboer 2000; Pariante and Miller 2001). Glucocorticoid receptor, a member of the nuclear receptor superfamily proteins, binds glucocorticoids in the cytoplasm and then translocates into the nucleus to work as a transcription factor, resulting in inhibition of secretion and synthesis of both corticotropin-releasing hormone (CRH) and adrenocorticotropic hormone (ACTH). In addition to the role on the HPA axis, GR expressed broadly throughout the brain is thought to modulate various neural functions such as learning and memory (Karst et al 2000; Lupien et al 2005). In response to stress, GR is associated with stress-induced effects on the brain, including shrinkage of neural dendrites, suppressed neurogenesis, and reduced serotonin metabolism (Lopez et al 1998; McEwen 2000; Sapolsky et al 2000; De Kloet 2003).

There are two protein isoforms of GR, GRα and GRβ, produced by alternative splicing. In contrast to GRα, which exerts glucocorticoid effects, GRβ is not able to bind glucocorticoids and is thought to form a heterodimer with GRα to exert a dominant-negative effect on GRα-mediated transcription (Oakley et al 1999; Vottero and Chrousos 1999). The proposed role for GRβ can explain the finding that enhanced expression of GRβ was associated with glucocorticoid resistance in allergic disease (Bamberger et al 1995; Leung et al 1997; Sousa et al 2000; Webster et al 2001). In addition, GRβ was reported to inhibit apoptosis induced by glucocorticoids in vitro (Strickland et al 2001), although the biological function of GRβ remains controversial (Carlstedt-Duke 1999; Vottero and Chrousos 1999).

Recently, reduced expression of GRα messenger RNA (mRNA) on postmortem brains has been reported in the distinct regions of the cortex and hippocampus of major depressive disorder and bipolar disorder brains (Webster et al 2002; Knable et al 2004; Perlman et al 2004). In line with aberrant expression of GRα mRNA in the brain, lymphocytes of depressed patients showed reduced response to dexamethasone (Wodarz et al 1991, 1992; Calfa et al 2003) and a reduced number of glucocorticoid binding sites (Gormley et al 1985; Whalley et al 1986; Yehuda et al 1993), although the lack of alteration in glucocorticoid binding sites was also reported (Wassef et al 1990; Rupprecht et al 1991). These previous findings strongly support the important role of GR in the pathophysiology of mood disorders. To date, there have been no direct measurements of GRα mRNA and GRβ mRNA on peripheral blood cells of mood disorder patients. Moreover, postmortem brain studies lack information on plasma cortisol concentration and the HPA axis activity assessed by the DEX/CRH test. It is difficult to determine whether the reduced GRα expression is recognized only in the depressive state or continues after recovery from the depressive state, in other words, state-dependent or trait-dependent.

The aim of this study was to evaluate GRα mRNA and GRβ mRNA levels in the peripheral white blood cells of individuals with major depressive disorder and bipolar disorder. Furthermore, to examine whether altered GR mRNA expression is state-dependent or trait-dependent, mood disorder patients in remission and first-degree relatives of mood disorder patients were also assessed.

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Table 1. Summarized Profile of Antidepressant Medication for Longitudinally Followed Subjects with Major Depressive Disorder and Bipolar Disorder

	MDD				BPD			
	Treatment Period (Months)				Treatment Period (Months)			
Anti-depressant Medication	0	2	4	6	0	2	4	6
Tricyclic Antidepressant	3				1			
Tricyclic Antidepressant + SSRI	3	2	2	1				
Tricyclic Antidepressant + SNRI	2	2						1
SSRI	2	7	6	6	4	3	2	1
SNRI	2	2	2	2	2	1	1	
SSRI + SNRI		2		2	1		1	
Tetracyclic Antidepressant	3	3	1		1	4	2	2
No Antidepressant	5	1			4	2	3	4
Total Number	20	19	11	11	13	10	9	8

MDD, major depressive disorder; BPD, bipolar disorder; SSRI, selective serotonin reuptake inhibitor; SNRI, serotonin-noradrenaline reuptake inhibitor.

Methods and Materials

Subjects

Major depressive and bipolar disorder patients were diagnosed according to the criteria in the *Diagnostic and Statistical Manual of Mental Disorders, Fourth Edition* (DSM-IV) (American Psychiatric Association 1994). These included both outpatients and inpatients of the Division of Neuropsychiatry of the Yamaguchi University Hospital. The extent of depressive state was assessed by a 21-item Hamilton Depression Rating Scale (HDRS). Subjects were regarded as under a current depressive state when they showed a score of more than 20 on HDRS and met the DSM-IV criteria for major depressive episode. Subjects were regarded to be in remission when they showed a score of less than 6 on HDRS and did not show any symptoms of the major depressive episode in the DSM-IV criteria for more than 2 months. A group of individuals with mood disorder in a current depressive state was assessed every 2 months for 6 months to investigate gene expression alteration during recovery from depressive states. Antidepressant medications for these subjects are summarized in Table 1. Individuals were excluded from the present study when they showed abnormal physical examinations or abnormal results for routine medical laboratory tests such as a complete blood count and renal, liver, and thyroid function. Female subjects who were pregnant or took oral contraceptives were also excluded. First-degree relatives who had no significant current or past medical or neurological illness, significant alcohol or drug abuse, and past or current Axis I psychiatric illness were enrolled (Table 2). All normal control subjects were screened to exclude significant current or past medical or neurological illness, significant alcohol or drug abuse, and past or current Axis I psychiatric illness. This protocol was approved by the Institutional Review Board of Yamaguchi University Hospital. Informed written consent was obtained for all subjects.

Blood Sample Preparation

Blood was obtained by venipuncture between 10:00 A.M. and 11:00 A.M. and processed to determine plasma cortisol concentration and total RNA purification.

RNA Isolation and Complementary DNA Synthesis

Total RNA was prepared from blood samples using QIAamp RNA Blood Mini kit (Qiagen, Chatsworth, California). The total RNA yield was determined by OD260. One microgram of total RNA was used for complementary DNA (cDNA) synthesis by random hexamer and Omniscript reverse transcriptase (Qiagen). The cDNA was stored at −80°C until use.

Real-Time Quantitative Polymerase Chain Reaction

Real-time quantitative polymerase chain reaction (PCR) was performed on cDNA with LightCycler (Roche Molecular Biochemicals, Germany) using the QuantiTect SYBR Green PCR kit (Qiagen) according to the manufacturer's manual. Polymerase chain reaction conditions were 15 minutes at 95°C, 35 to 45 cycles of 15 seconds at 95°C, 20 seconds at 55°C, and 10 seconds at 72°C. Cycle number was optimized for each primer set corresponding to GRα, GRβ, and gylceraldehyde-3-phosphate dehydrogenase (GAPDH). Used primer sets were 5-gaactggcagcggttttatc-3 and 5-tctcggggaattcaatactca-3 for GRα, 5-ccattgtcaagagggaagga-3 and 5-tgtgtgagatgtgctttctgg-3 for GRβ, and 5-cagcctcaagatcatcagca-3 and 5-tgtggtcatgagtccttcca-3 for GAPDH. Amplification of the single PCR product was confirmed by monitoring the dissociation curve. Amplification curves were visually inspected to set a suitable baseline range and threshold level. To generate standard curves, different concentrations of cDNA made from total RNA isolated from human lymphoma cell line Jurkat cells were used in each PCR reaction. The number of cycles required to reach the threshold fluorescence level was scored and used for generating standard curves and interpolating mRNA concentration levels. The relative quantification method was employed for quantification of target molecules according to the manufacturer's protocol, in which the ratio between the amount of target molecule and a reference molecule within the same sample was calculated. At a minimum, all measurements were performed in duplicate. The GAPDH mRNA level was used

Table 2. Demographic and Clinical Characteristics of Subjects

		Patients					
		MDD		BPD		Relatives	
	Control Subjects $n = 31$	Depressed $n = 18$	Remission $n = 38$	Depressed $n = 13$	Remission $n = 35$	MDD $n = 17$	BPD $n = 15$
Mean Age (years)	49.9 ± 1.7	52.9 ± 3.9	58.3 ± 2.1	55.5 ± 3.7	52.9 ± 2.4	48.8 ± 3.6	44.7 ± 4.8
Gender (Female/Male)	15/16	9/9	14/24	11/2	29/6	13/4	10/5
HDRS		26.9 ± 2.0	3.7 ± .32	24.6 ± 1.1	3.2 ± .33		
Serum Cortisol (μg/dL)	8.8 ± .79	10.9 ± 3.5	10.2 ± .76	9.4 ± 2.1	11.5 ± .97		

Includes subjects with bipolar disorder, major depressive disorder, first-degree relatives of BPD and MDD, and normal control subjects. MDD, major depressive disorder; BPD, bipolar disorder; HDRS, Hamilton Depression Rating Scale.

for normalization. Expression value was normalized by dividing the mean of the value of control subjects.

Plasma Cortisol Determination

Plasma cortisol concentration was measured with radioimmunoassay by the laboratory of SRL Corporation (Tokyo, Japan).

DEX/CRH Test

A subgroup of subjects in a current depressive state underwent the DEX/CRH test as previously reported with minor modifications (Heuser et al 1996). Mood disorder patients were pretreated with an oral dose of 1 mg of dexamethasone (DEX) (Dexamethasone, Asahikasei Pharmaceutical Corporation, Tokyo, Japan) at 11:00 P.M. The next day, intravenous cannulation was carried out at 12:30 P.M. and 100 μg of human CRH (hCRH, Mitsubishi Pharma Corporation, Tokyo, Japan) was administered intravenously at 1:00 P.M., immediately after the first blood collection. Blood specimens were drawn through the intravenous catheter 15 minutes, 30 minutes, 60 minutes, and 120 minutes later. Blood samples were immediately centrifuged and stored at −20°C. Plasma levels of cortisol and ACTH were measured with radioimmunoassay (SRL Corporation). We defined nonsuppressors as those individuals whose post-DEX plasma cortisol levels were more than 5 μg/dL.

Data Analysis

Data are presented as means ± standard error of mean (SEM) unless otherwise specified. Distributions for each variable were examined for normality using Shapiro and Wilk's test. When homogeneity of variances and a normal distribution of data were detectable, one-way analysis of variance (ANOVA) and post hoc test (Tukey test) were used for statistical analysis. When significant deviations from normality were found ($p < .05$), nonparametric statistics were applied. When deviation from normality and lack of homogeneity of variances occurred, Kruskal-Wallis one-way analysis of variance was used for statistical analysis and then, if significant, the Steel-Dwass test was used for group comparisons. The Spearman rank correlation was calculated to assess the correlation between data. Two group comparisons, such as the effect of antidepressant usage or gender on GR mRNA expression, were performed using the Student t test. For categorical variables, the chi-square test was used. The analysis of covariance (ANCOVA) using age as a covariate was performed to assess GR mRNA expression levels between suppressors and nonsuppressors of the DEX-CRH test. For all statistical analysis, $p < .05$ was considered significant.

Results

The mean ages were not significantly different between major depressive disorder patients, bipolar disorder patients, and normal control subjects ($F = 1.95$, $df = 4,131$, $p = .106$) (Table 2). Regarding the gender distribution, bipolar disorder patients showed a larger ratio of female to male ($X^2 = 12.7$, $df = 4$, $p = .013$). Levels of plasma cortisol did not differ between major depressive disorder patients, bipolar disorder patients, and normal control subjects ($F = .92$, $df = 4,58$, $p = .456$). Real-time PCR revealed that the expression level of GRα mRNA was decreased in major depressive disorder patients in a current depressive state and in remission, compared with normal control subjects ($F = 8.13$, $df = 4,131$, $p < .0001$, post hoc $p = .028$, and $p = .011$, respectively) (Figure 1). The levels of GRα mRNA expression of major depressive disorder patients showed no significant difference between the depressive state and remission ($p = .382$).

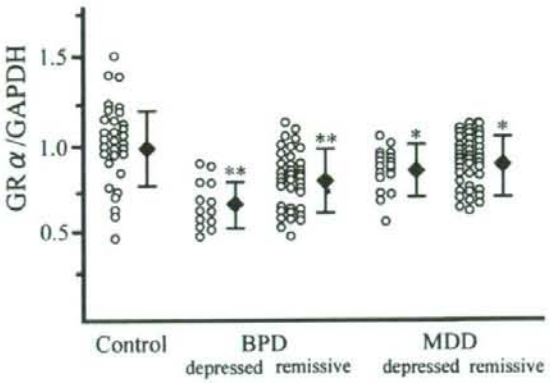

Figure 1. Quantitative RT-PCR revealed that reduced GRα mRNA expression was shown in major depressive disorder patients in a current depressive state ($n = 18$), major depressive disorder patients in remission ($n = 38$), in bipolar disorder patients in a current depressive state ($n = 13$), and in bipolar patients in remission ($n = 35$) compared with normal control subjects ($n = 31$). Values are mean ± standard error. $^*p < .05$, $^{**}p < .01$. RT-PCR, reverse transcription polymerase chain reaction; mRNA, messenger RNA; MDD, major depressive disorder; BPD, bipolar disorder.

Reduced GRα mRNA expression was also recognized in bipolar disorder patients in a current depressive state as well as in remission (post hoc $p < .0001$ and $p = .0005$, respectively) (Figure 1). The GRα mRNA expression levels of bipolar disorder patients showed no significant difference between depressive state and remission ($p = .238$). There was no significant correlation between GRα mRNA level and plasma cortisol concentration of normal control subjects, major depressive disorder patients, and bipolar disorder patients ($r = -.010$, $p = .967$; $r = .037$, $p = .508$; $r = .269$, $p = .238$, respectively). Gender difference did not produce significant effects on GRα mRNA expression of normal control subjects ($t = -.641$, $df = 29$, $p = .526$), major depressive disorder patients ($t = -.266$, $df = 54$, $p = .792$), and bipolar disorder patients ($t = -.291$, $df = 46$, $p = .772$). No significant correlation between age and GRα mRNA level was shown in normal control subjects ($r = .144$, $p = .439$), major depressive disorder patients in a current depressive state ($r = -.108$, $p = .671$), major depressive disorder patients in remission ($r = -.111$, $p = .508$), bipolar disorder patients in a current depressive state ($r = -.278$, $p = .357$), and bipolar disorder patients in remission ($r = -.004$, $p = .839$).

In contrast to GRα, there was no significant difference of GRβ mRNA expression between mood disorder patients in a current depressive state and normal control subjects ($F = .27$, $df = 2,59$, $p = .762$) (Figure 2). Also, no significant difference in GRβ mRNA expression was found between the first-degree relatives of mood disorder patients and normal control subjects ($F = 1.71$, $df = 2,58$, $p = .190$) (data not shown). A strong negative correlation was found between the expression levels of GRα mRNA and of GRβ mRNA of control individuals ($r = -.463$, $p = .009$) (Figure 3). However, there were no significant correlations between the expression levels of GRα mRNA and of GRβ mRNA of both major depressive disorder patients in a current depressive state ($r = .169$, $p = .502$) and bipolar disorder patients in a current depressive state ($r = .349$, $p = .243$) (Figure 3). There was no significant correlation between GRβ mRNA level and plasma cortisol concentration of normal control subjects, major depressive disorder patients, and bipolar disorder patients ($r = .242$,

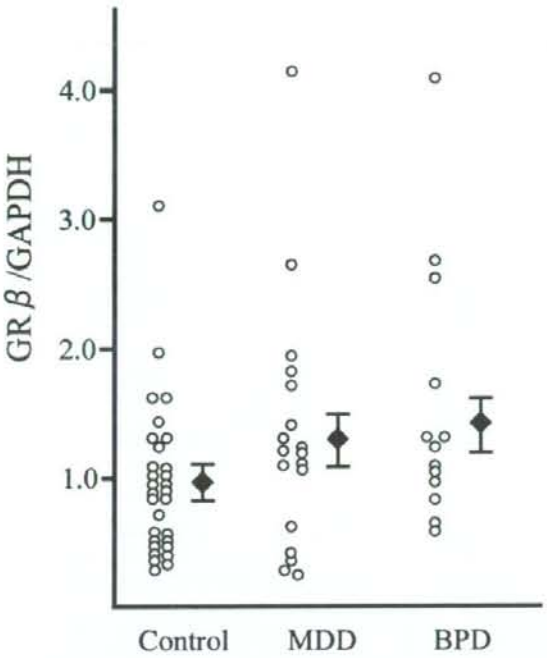

Figure 2. Quantitative RT-PCR revealed that GRβ mRNA levels of subjects with major depressive disorder (n = 18) and bipolar disorder (n = 13) did not show significant differences from that of normal control subjects (n = 31). Values are mean ± standard error. RT-PCR, reverse transcription polymerase chain reaction; mRNA, messenger RNA; MDD, major depressive disorder; BPD, bipolar disorder.

p = .304; r = −.632, p = .253; r = .400, p = .600, respectively). Gender difference did not produce a significant effect on GRβ mRNA expression of normal control subjects (t = .968, df = 29, p = .341), major depressive disorder patients in a current depressive state (t = .265, df = 16, p = .794), and bipolar disorder patients in a current depressive state (t = −.794, df = 11, p = .444). No significant correlation between age and GRβ mRNA level was shown in normal control subjects (r = −.136, p = .466), major depressive disorder patients in a current depressive state (r = −.157, p = .533), and bipolar disorder patients in a current depressive state (r = −.154, p = .614).

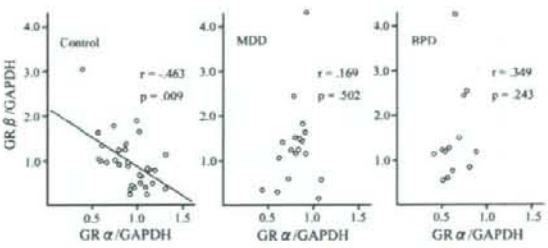

Figure 3. Significant correlation between GRα mRNA levels and GRβ mRNA levels was recognized in normal control subjects (n = 31) but not in major depressive disorder patients (n = 18) or bipolar disorder patients in a current depressive state (n = 13). mRNA, messenger RNA; MDD, major depressive disorder; BPD, bipolar disorder.

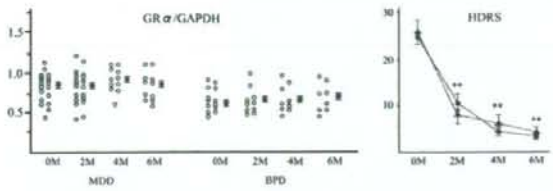

Figure 4. During recovery from a depressive state, stable reduction of GRα mRNA expression was shown at 0 (n = 20), 2 (n = 19), 4 (n = 11), and 6 months (n = 11) in subjects with major depressive disorder and at 0 (n = 13), 2 (n = 10), 4 (n = 9), and 6 months (n = 8) in subjects with bipolar disorder. HDRS values of major depressive patients (circle) and of bipolar disorder patients (triangle) were significantly reduced at 2, 4, and 6 months compared with 0 months. Values are mean ± standard error. ** p <0.01. mRNA, messenger RNA; MDD, major depressive disorder; BPD, bipolar disorder; HDRS, Hamilton Depression Rating Scale.

To investigate whether GRα mRNA levels vary during recovery from depressive state, individuals with major depressive disorder (n = 20) and individuals with bipolar disorder (n = 13) in a current depressive state were assessed for GRα mRNA expression every 2 months for 6 months. No significant difference of GRα mRNA level was detected between 0 (n = 20), 2 (n = 19), 4 (n = 11), and 6 (n = 11) months in major depressive disorder patients (F = 1.38, df = 3,57, p = .258). No significant difference of GRα mRNA level was detected between 0 (n = 13), 2 (n = 10), 4 (n = 9), and 6 (n = 8) months in bipolar disorder patients (F = .32, df = 3,36, p = .810). Hamilton Depression Rating Scale values were significantly reduced at 2 months in major depressive disorder patients and bipolar disorder patients (F = 47.2, df = 3,57, p < .0001, post hoc p < .0001 and F = 24.5, df = 3,36, p < .0001, post hoc p < .0001, respectively) (Figure 4).

Next, to examine whether reduced GRα expression is present in trait-dependent change of mood disorder, GRα mRNA expressions were determined in first-degree relatives of major depressive disorder patients (n = 17) and bipolar disorder patients (n = 15). Reduced GRα mRNA expression was recognized in the first-degree relatives of bipolar disorder patients but not in the first-degree relatives of major depressive disorder patients (F = 3.30, df = 2,60, p = .043, post hoc p = .040, and p = .968, respectively) (Figure 5). There were no significant differences in GRα mRNA expression regarding gender of first-degree relatives of major depressive disorder patients (t = .157, df = 3, p = .205) and those of bipolar disorder patients (t = 1.05, df = 13, p = .312). No significant correlation between age and GRα mRNA level was shown in first-degree relatives of major depressive disorder patients (r = −.283, p = .270) and those of bipolar disorder patients (r = .285, p = .303).

To examine whether the reduction of GRα mRNA expression in mood disorders would have an influence on the HPA axis activity, GRα and GRβ mRNA levels of mood disorder patients in a current depressive state were compared between suppressors (n = 13, 10 major depressive disorder patients and 3 bipolar disorder patients) and nonsuppressors (n = 16, 8 major depressive disorder patients and 8 bipolar disorder patients) of the DEX/CRH test. The mean age of nonsuppressors was significantly higher than suppressors (59 ± 3.3 vs. 44 ± 5.5; t = 2.47, df = 29, p = .020) with similar gender distribution (X^2 = 3.84, df = 1, p = .11), which is consistent with a previous report (Heuser et al 1994). Thus, we applied analysis of covariance using age as a covariate, finding no significant difference in GRα and GRβ mRNA expression level between suppressors and nonsuppres-

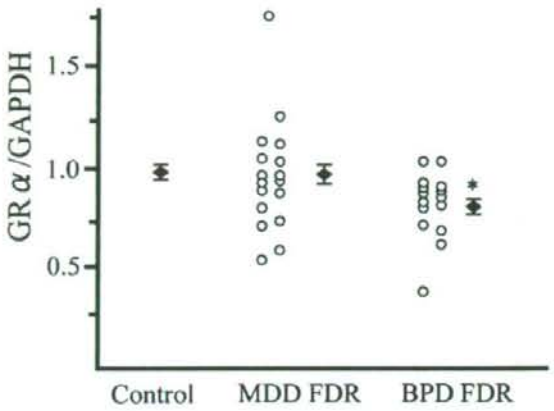

Figure 5. Quantitative RT-PCR revealed that GRα mRNA expression was reduced in first-degree relatives of bipolar disorder patients ($n = 15$) but not in first-degree relatives of major depressive disorder patients ($n = 17$) compared with normal control subjects ($n = 31$, data shown in Figure 1). Values are mean ± standard error. * $p < .05$. RT-PCR, reverse transcription polymerase chain reaction; mRNA, messenger RNA; MDD FDR, first-degree relatives of major depressive disorder; BPD FDR, first-degree relatives of bipolar disorder patients.

sors ($F = 1.04$, $df = 1,26$, $p = .319$; $F = 1.97$, $df = 1,26$, $p = .179$, respectively) (Figure 6).

Finally, to examine the effect of antidepressants on GRα mRNA expression, bipolar disorder patients in remission were divided into two groups regarding antidepressant medication and assessed for GRα mRNA expression. No significant difference in GRα mRNA was shown between bipolar disorder patients in remission ($n = 18$) medicated with both antidepressants and mood stabilizers and those ($n = 18$) medicated with mood stabilizers only ($t = 1.30$, $df = 1,34$, $p = .20$). Also, seven drug-free patients (bipolar disorder $n = 1$; major depressive disorder $n = 6$) showed significant GRα mRNA reduction compared with normal control subjects ($t = 2.14$, $df = 1,35$, $p = .038$).

Discussion

In this study, we demonstrated that GRα mRNA level reduction occurs in peripheral blood cells of individuals with major depressive disorder and bipolar disorder, in both a depressive state as well as in remission. Reduced GRα mRNA expression was also shown in first-degree relatives of bipolar disorder patients but not in those of major depressive disorder patients. Based on these and previous findings that GRα mRNA reduction occurred in the cerebral cortex, hippocampus, and amygdala of mood disorder brains (Webster et al 2002; Knable et al 2004; Perlman et al 2004), we propose that the GRα mRNA level in mood disorder patients is decreased in multiple systems. Glucocorticoid receptor plays multiple roles in the brain, such as modulation of neural activity and regulation of the HPA axis, as well as in the immunological system (Riccardi et al 2002). The GRα mRNA reduction in the peripheral blood cells of mood disorder patients may be associated with the immunological alteration of mood disorder patients such as increased plasma concentration of interleukin-1 (IL-1) and interleukin-6 (IL-6) (Maes et al 1995; Owen et al 2000; Anisman and Merali 2003), although there was controversy about immunological alteration of mood disorder patients.

The present findings of GRα mRNA reduction in mood disorder patients in remission and continuous GRα mRNA reduction during recovery from a depressive state show that altered GRα mRNA levels are not restricted to depressive states but continue after recovery. Furthermore, first-degree relatives of bipolar disorder patients also showed reduced GRα mRNA levels. These results suggest that GRα mRNA reduction is not a state-dependent finding but a trait-dependent finding of mood disorder, especially in bipolar disorder. Together with previous findings that remissive bipolar disorder patients and first-degree relatives of bipolar disorder patients showed disturbed GR function assessed by cortisol concentration after the DEX/CRH test (Holsboer et al 1995; Watson et al 2004), first-degree relatives of bipolar disorder patients as well as remissive bipolar disorder patients may have GR dysregulation.

Although GRα mRNA level could be influenced by plasma cortisol, plasma cortisol concentrations at 10:00 A.M. were not different between individuals with mood disorders and normal control subjects, which is consistent with previous reports (Young et al 1994, 2001). Both GRα mRNA and GRβ mRNA levels did not correlate with plasma cortisol concentrations of normal control subjects as well as mood disorder patients, suggesting that GRα mRNA and GRβ mRNA levels were not simply determined by plasma cortisol concentrations. One cannot deny the possibility that altered baseline plasma cortisol concentrations of mood disorder patients, which was not determined in the present study, may reduce GRα mRNA levels. However, there have been inconsistent reports that evening baseline plasma concentrations of mood disorder patients either increased (Young et al 1994, 2001) or decreased (Vythilingam et al 2004). Furthermore, increased glucocorticoid concentrations do not result in the down-regulation of GRα, since a normal number of glucocorticoid-binding sites were shown in recovered depressive patients with sustained high plasma cortisol concentrations (Hunter et al 1988) and Cushing's disease patients (Invitti et al 1999; Huizenga et al 2000). Thus, our finding of GRα mRNA reduction in mood disorder patients may not be a direct consequence of elevated cortisol concentrations.

Although most individuals in our study with mood disorders were on antidepressant medication, which can influence GRα mRNA expression, drug-free patients also showed GRα mRNA reduction. Furthermore, GRα mRNA reduction of bipolar disor-

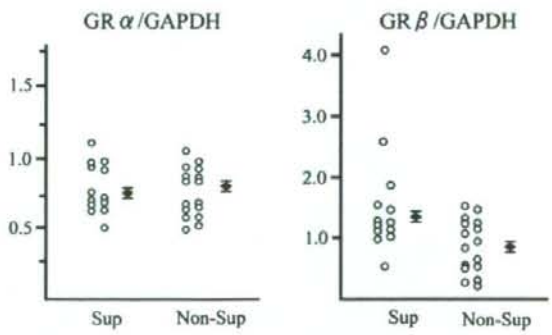

Figure 6. No significant difference in GRα mRNA or GRβ mRNA expression of mood disorder patients was shown between suppressors ($n = 13$) and nonsuppressors ($n = 16$) of the DEX/CRH test. mRNA, messenger RNA; DEX/CRH, dexamethasone/corticotropin-releasing hormone; Sup, suppressors; Non-Sup, nonsuppressors.

der patients in remission occurred regardless of antidepressant medication. These results are consistent with the report of the lack of statistical difference between GRα mRNA levels in the brain of subjects on antidepressant medication and subjects free of antidepressant medication (Webster et al 2002). In vivo and in vitro studies on the effect of antidepressants on GRα expression showed an increase or unaltered expression of GRα (Seckl and Fink 1992; Barden et al 1995; Pariante et al 1997; Okugawa et al 1999; Vedder et al 1999; Pariante and Miller 2001), although there was a report that some antidepressants reduced GRα mRNA in blood cells (Heiske et al 2003). Thus, antidepressant medication alone might not be a major determinant of GRα mRNA reduction in the peripheral blood cells. This is supported by the present result that first-degree relatives of bipolar disorder patients with no exposure to antidepressant medication also showed a reduction in GRα mRNA.

The present result showed no significant difference in GRα and GRβ mRNA expression levels between suppressors and nonsuppressors of the DEX/CRH test in mood disorder patients. Nonsuppression of the DEX/CRH test is thought to be indicative of a blunted response to increased glucocorticoid via GRα-mediated transcription (Barden et al 1995; Holsboer 2000; Pariante and Miller 2001). The finding of unaltered GRβ expression between suppressors and nonsuppressors is not consistent with the proposed function for GRβ as an antagonist for GRα-mediated transcription. There was a report that GRβ does not act as an antagonist for GRα-mediated transcription (Lange et al 1999). Thus, the biological function of GRβ remains controversial (Carlstedt-Duke 1999; Vottero and Chrousos 1999). Both GRβ mRNA and GRβ protein have been repeatedly reported to be extremely low in lymphocytes, as well as in the brain, compared with GRα (DeRijk et al 2003; Pedersen and Vedeckis 2003). Together with the present results of unaltered GRβ expression in mood disorder patients, GRβ may not play an important role in the pathogenesis of mood disorders. However, it is possible that GRβ may exert some effects on the dysregulation of the HPA axis in mood disorder patients.

It is not surprising there was no detectable difference in GRα mRNA regardless of the results of the DEX/CRH test, which is indicative of GRα-mediated transcription, because GRα-mediated transcription is modified by many factors including GR phosphorylation, nuclear localization, and interaction with other molecules such as AP1, NFκ-B, and GRβ (Oakley et al 1999; Hayashi et al 2004). Moreover, aberrant HPA axis function of mood disorder is thought to be related to many neurotransmitters and hormones such as arginine vasopressin (AVP) (Holsboer and Barden 1996; Holsboer 2001), gamma-aminobutyric acid (GABA), and glutamate (Herman et al 2004). Furthermore, a recent report has shown discrepancies between the GR mRNA level, GR protein level, and GR function in peripheral blood mononuclear cells (Torrego et al 2004). Future studies should examine whether GRα mRNA reduction is associated with reduced GRα protein expression and disturbed GRα function.

Another finding of our study is the inverse correlation between the amount of GRα and GRβ mRNA in normal control subjects but not in mood disorder patients. The GRβ mRNA is produced from pre-mRNA common to GRα mRNA by alternative splicing associated with SRp30c (Xu et al 2003). Although regulatory mechanisms of alternative splicing remains to be clarified, the present result suggests that GRα may suppress GRβ production by regulating the alternative splicing in normal control subjects. If this hypothesis is valid, the lack of a significant correlation between GRα and GRβ recognized in mood disorder patients suggests aberrant alternative splicing associated with GRα-mediated transcription in mood disorder patients. It is also possible that an alternative splicing mechanism itself is disturbed in mood disorder patients (Lee and Irizarry 2003).

Although the pathological significance of reduced GRα mRNA expression in mood disorder patients is a problem, heterozygous mice of the GR-deficient mouse showed normal behavior at baseline with enhanced helplessness and despair in response to stress and nonsuppression to the DEX/CRH test (Ridder et al 2005). Thus, reduced expression of GRα mRNA could be one factor that leads individuals to be susceptible to stress and mood disorders.

TM and HF contributed equally to this work.

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Research report

Increased expression of splicing factor SRp20 mRNA in bipolar disorder patients

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Abstract

Background: Variations and defects in alternative splicing are well known to be associated with a variety of human diseases and the stress response. We previously reported a decrease in glucocorticoid receptor (GR) α, but not GRβ in mood disorder patients, suggesting an aberrant alternative splicing mechanism. To examine whether altered RNA splicing may underlie the pathophysiology of mood disorder, we evaluated the expression of a variety of SR protein splicing factors, a family of proteins indispensable for proper alternative splicing, in mood disorder patients.

Methods: We used quantitative real-time PCR to measure expressions of SRp20, SRp30c, SC35, SRp40, SRp46, SRp54, SRp55, SRp75, ASF/SF2, and 9G8 mRNA in peripheral white blood cells of 33 mood disorder patients during a depressive episode. In addition, the expressions of SRp20 and SC35 mRNA were quantified for 78 mood disorder patients in a remissive state, and 32 the first-degree relatives of these mood disorder patients.

Result: A significant correlation was observed between SRp30c and the GRβ/GRα ratio in control subjects, but not in mood disorder patients. Increased expression of SRp20 but not SRp30c mRNA was observed in bipolar disorder patients in both the depressive and remissive states. Major depressive disorder patients did not show any significant change in mRNA levels of SR proteins.

Limitation: Subjects were Japanese adults. Patient treatment was not standardized.

Conclusions: These results suggest that aberrant alternative splicing machinery caused by increased SRp20 mRNA expression would be associated with the pathophysiology of bipolar disorder.

Keywords: Alternative splicing; SR protein; SRp20; Mood disorder; Bipolar disorder

1. Introduction

Mood disorder patients often exhibit hyperactivity of the hypothalamic–pituitary–adrenal (HPA) axis (Pariante and Miller, 2001; Pariante, 2004; de Kloet et al., 2005). One of the plausible mechanisms to lead the HPA

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axis to hyperactivity is the dysfunction of negative feedback regulation in which the glucocorticoid receptor (GR) plays crucial roles (Holsboer, 2000; Pariante and Miller, 2001; de Kloet et al., 2005). Several lines of evidences have indicated the decrease in GR expressions of mood disorder patients in distinct regions of the cortex and hippocampus (Webster et al., 2002; Knable et al., 2004; Perlman et al., 2004), as well as in peripheral blood cells (Pariante, 2004; Matsubara et al., 2006).

GR has several isoforms produced by alternative splicing, two of which (GRα and β) have been the focus of most studies because of their relative abundance (Lu and Cidlowski, 2006). Our previous study indicated that the levels of GRα mRNA were decreased in the peripheral blood cells of mood disorder patients, whereas GRβ mRNA levels were unaltered (Matsubara et al., 2006). Additionally, although healthy control subjects showed a significant inverse correlation between levels of GRα and GRβ mRNAs, neither major depressive disorder (MDD) nor bipolar disorder (BPD) patients showed such correlations. These findings suggest the possibility of a defective alternative splicing mechanism in mood disorder patients.

Alternative splicing is widely regarded as a major source of diversity in the human proteome. Recently, defective alternative splicings were reported to be associated with many neuropsychiatric diseases (Grabowski and Black, 2001; Lee and Irizarry, 2003; Garcia-Blanco et al., 2004). These findings suggest that possible deficits in the alternative splicing mechanism lead to change in gene expression in patients with various psychiatric diseases including mood disorders.

One of the best characterized molecules regulating splicing of RNA transcript is the family of SR protein splicing factors (Graveley, 2000; Black, 2003; Lareau et al., 2007). SR proteins are critical components of constitutive splice site recognition and often bind specifically near alternative splice sites to favor their selection (Zheng, 2004). Among SR proteins, SRp30c is involved in the alternative splicing producing GRβ mRNA from GR pre-mRNA (Xu et al., 2003). Also, SC35 is thought to be increased by stress, and facilitate the shift to alternative splicing of acetyl cholinesterase pre-mRNA in mouse brain (Meshorer et al., 2005). These findings suggest that the aberrant expression and/or function of SR protein might be involved in the pathophysiology of mood and stress-related disorders. However, there are no reports examining the expression of SR proteins in mood disorder patients.

The aim of this study is to examine whether the expressions of SR protein splicing factor mRNAs are altered in the peripheral white blood cells of patients with mood disorders in both the depressive and remissive states, as well as in the first-degree relatives of mood disorder patients. In addition, we examined whether there are any correlations between SR protein mRNA levels, GRα and β mRNA levels, the ratio of GRβ mRNA to GRα mRNA (GRβ/GRα) and serum cortisol concentrations.

2. Methods and materials

2.1. Subjects

A demographic summary of healthy control subjects ($n=28$), mood disorder subjects ($n=33$ in a depressive state, $n=78$ in a remissive state), and the first-degree relatives of the mood disorder subjects ($n=32$) are shown in Table 1. MDD and BPD patients were diagnosed according to the criteria in the Diagnostic and Statistical Manual of Mental Disorders, fourth edition (DSM-IV). These included both outpatients and inpatients seen by the Division of Neuropsychiatry of the Yamaguchi University Hospital. The severity of the depressive state was assessed by a 21-item "Hamilton Depression Rating Scale" (HDRS). Subjects with an HDRS score of more than 18 were regarded as being in a depressive state, and those with a score of less than 6 and who showed no symptoms of a major depressive episode according to the DSM-IV criteria for more than 2 months were regarded as being in a remissive state. Individuals were excluded from the present study if they had abnormal physical examinations or abnormal results for routine medical laboratory tests such as a complete blood count, renal, liver or thyroid functions. Female subjects who were pregnant or took oral contraceptives were also excluded. All healthy controls subjects and the first degree relatives were genetically unrelated residents living in Japan without either mental past histories, and controls and patients were all of Japanese ethnicity and there is no significant population stratification in Japanese reported in several groups (Kakiuchi et al., 2003; Yamada et al., 2004). This protocol was approved by the Institutional Review Board of Yamaguchi University Hospital. Informed written consent was obtained for all subjects.

2.2. Blood sample preparation

Blood was obtained by venipuncture between 10:00 A.M. and 11:00 A.M., and processed to determine serum cortisol concentration and for total RNA purification.

2.3. RNA isolation and cDNA synthesis

Total RNA was prepared from peripheral white blood cells using QIAamp RNA Blood Mini kit (Qiagen,

Chatsworth, CA). The total RNA yield was determined by OD260. One microgram of total RNA was used for cDNA synthesis by random hexamer and Omniscript reverse transcriptase (Qiagen). The cDNA was stored at −80 °C until further use.

2.4. Real-time quantitative PCR

Real-time quantitative polymerase chain reaction (PCR) was performed as described previously (Matsubara et al., 2006). Used primers sets were 5-TCTTGG AAACAATGGCAACA-3 and 5-CTCGGGGATCTT CAAATTCA-3 for SRp20, 5-ATATGCCCTGCGT AAACTGG-3 and 5-AGACCGAGACCGTGAGT AGC-3 for SRp30c, 5-CCCGGACTCACACCACAG-3 and 5-AGATCGGCTGCGAGACCT-3 for SC35, 5-GAGGCTTTGGTTTTGTGGAA-3 and 5-CGAGCCC TAGCATGTTCAAT-3 for SRp40, 5-GCTACGGCG GATCTCACTAC-3 and 5-TTGATGCAGAGCGAG AGCTA-3 for SRp46, 5-TCCAGACTCAGCAG TTGTGG-3 and 5-TGGTGCCAACAGAGACAAAG-3 for SRp54, 5-AAATACGGACCACCTGTTCG-3 and 5-CTTCACCTGCTTGTCGCATA-3 for SRp55, 5-GAT CCTGGAGGTGGATCTGA-3 and 5-CCACA AAGGTCTTTGCCATT-3 for SRp75, 5-TGCCTACA TCCGGGTTAAAG-3 and 5-CTGCTGTTGC TTCTGCTACG-3 for ASF/SF2, 5-CGCTGGCAAAG-GAGAGTTAG-3 and 5-CGAATTCCACAAAGG CAAAT-3 for 9G8, and 5-CAGCCTCAAGATCATC AGCA-3 and 5-TGTGGTCATGAGTCCTTCCA-3 for GAPDH. All measurements were performed at least in duplicate. GAPDH mRNA levels were used for normalization. Expression values for mood disorder subjects were normalized by the mean of the value for control subjects. Methods and results of the quantitative analysis of GRα and GRβ mRNA levels were described in detail in our previous report (Matsubara et al., 2006).

2.5. Serum cortisol determination

Serum cortisol concentration was measured via radioimmunoassay by the SRL Corporation (Tokyo, Japan).

2.6. Data analysis

Data are presented as mean ± standard error of the mean (SEM) unless otherwise specified. For statistical analyses, we used one-way analysis of variance (ANOVA) followed by the Dunnett test. Spearman's correlations were calculated to assess the correlation between data. Two group comparisons, such as the effects of mood stabilizer usage or gender on SR protein mRNA expression, were performed using the Student's *t*-test. For all statistical analyses, $p<0.05$ was considered significant.

3. Results

3.1. Increased SRp20 mRNA expression in BPD patients during depressive and remissive states

We first examined the expression of SR protein splicing factors, SRp20, SRp30c, SC35, SRp40, SRp46,

Table 1
Demographic and clinical characteristics of subjects

	Controls	Patients				Relatives	
		MDD		BPD		MDD	BPD
		Depressed	Remission	Depressed	Remission		
	$n=28$	$n=20$	$n=41$	$n=13$	$n=37$	$n=17$	$n=15$
Age (years)	50.0±1.8	52.3±3.5	57.3±2.2	55.5±3.5	53.2±2.3	48.8±3.5	44.7±4.7
Gender (male/female)	15/13	10/10	15/26	2/11	7/30	4/13	5/10
HDRS		25.9±1.9	3.2±0.2	24.6±1.1	2.9±0.2		
Serum cortisol (μg/dl)	8.6±0.8	10.3±1.3	11.6±1.1	10.9±4.5	10.5±0.8		
Medication							
No medication	28	3	4	1	0	17	15
SSRI	0	5	13	4	3	0	0
TCA	0	8	8	1	6	0	0
Li	0	0	0	4	21	0	0
VPA	0	0	0	7	16	0	0
CBZ	0	0	0	2	8	0	0

MDD, major depressive disorder; BPD, bipolar disorder; Relatives, first-degree relatives of MDD and BPD patients; HDRS, Hamilton Depression Rating Scale; SSRI, selective serotonin reuptake inhibitor; TCA, tricyclic antidepressant; Li, lithium; VPA, valproic acid; CBZ, carbamazepine.

SRp54, SRp55, SRp75, ASF/SF2 and 9G8 in mood disorder patients in the depressive state (Fig. 1A). SRp30c mRNA expression was not altered in either BPD or MDD patients, compared to healthy control subjects ($F=0.122$, df=2, 58, $p=0.885$). Unexpectedly, the expression of SRp20 mRNA was significantly increased in BPD patients, compared to healthy control subjects ($F=5.437$, df=2, 58, $p=0.007$, post-hoc $p=0.003$), whereas in MDD patients there was only a slight, non-significant increase in SRp20 mRNA expression (post-hoc $p=0.262$). There were no significant effects of gender on SRp20 mRNA expression in healthy control subjects ($t=0.839$, df=26, $p=0.409$), MDD patients ($t=0.281$, df=18, $p=0.782$), or BPD patients ($t=-0.276$, df=11, $p=0.787$) in the depressive state. There were also no significant correlations between SRp20 mRNA expression and age in healthy control subjects ($r=0.096$, $p=0.634$), MDD patients ($r=0.110$, $p=0.644$), or BPD patients ($r=-0.396$, $p=0.180$) in this state.

Next, we examined the expression of SRp20 mRNA in mood disorder patients in a remissive state (Fig. 1B). We also examined SC35 mRNA expression in these patients, because it has been reported that stress increased SC35 expression (Meshorer et al., 2005). BPD patients showed a significant increase in SRp20 mRNA expression, similar in extent to that in a depressive state ($F=3.999$, df=2, 103, $p=0.021$, post-hoc $p=0.012$), whereas MDD patients did not show any alteration in SRp20 mRNA expression (post-hoc $p=0.341$). SC35 mRNA expression was not altered in either BPD or MDD patients, compared to healthy control subjects ($F=0.210$, df=2, 103, $p=0.811$).

As the expression of SRp20 mRNA was increased in BPD patients in both the depressive and remissive states, we also examined the expression of SRp20 and SC35 mRNA in the first-degree relatives of mood disorder patients. However, the expression of both of these factors were similar to those of healthy control subjects (SRp20: $F=0.136$, df=2, 57, $p=0.873$; SC35: $F=0.461$, df=2, 57, $p=0.633$).

3.2. SR proteins and the HPA axis

We examined the relationships between the SRp30c mRNA expression, which is reported to be associated with alternative splicing of the GR transcript (Xu et al., 2003), and the expression of GRα mRNA, GRβ mRNA, the GRβ/GRα ratio, and serum cortisol concentration. There was a significant correlation between the expression of SRp30c mRNA and GRβ/GRα in healthy control subjects, but not in either group of mood disorder patients in the depressive state (Fig. 2). SRp30c mRNA expression did not show any significant correlation with serum cortisol concentrations in healthy control subjects ($r=0.236$, $p=0.345$), MDD patients ($r=-0.092$, $p=0.813$), or BPD patients ($r=-0.051$, $p=0.935$) in the depressive state.

Unexpectedly, there was also a significant correlation between SRp20 mRNA expression and GRβ mRNA expression in MDD patients in a depressive state (Fig. 3)

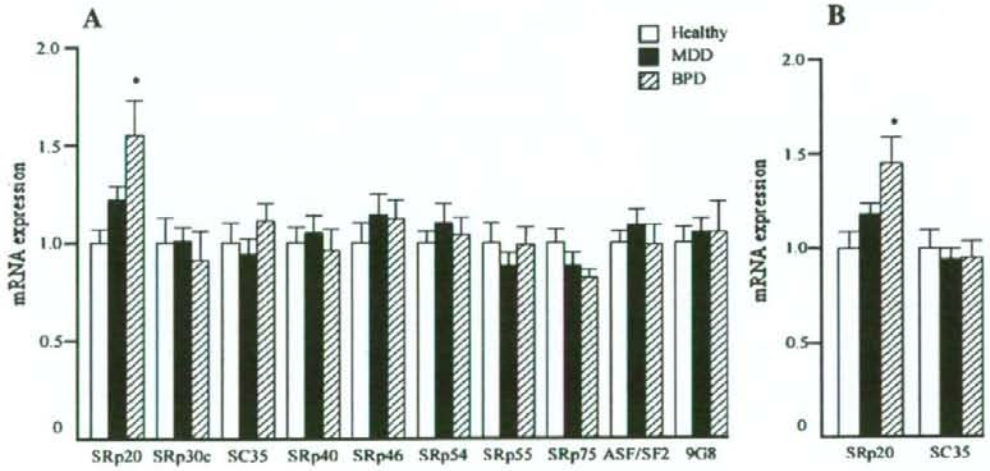

Fig. 1. The mRNA expression of SR protein splicing factors in mood disorder patients. A. Quantitative real-time PCR revealed an increased SRp20 mRNA expression only in BPD patients in a depressive state ($n=13$) compared to healthy control subjects ($n=28$). B. The expression of SRp20 mRNA was increased in BPD patients (n=37) in a remissive state compared to healthy control subjects ($n=28$). MDD, major depressive disorder; BPD, bipolar disorder. Values are mean±standard error. *$p<0.05$.

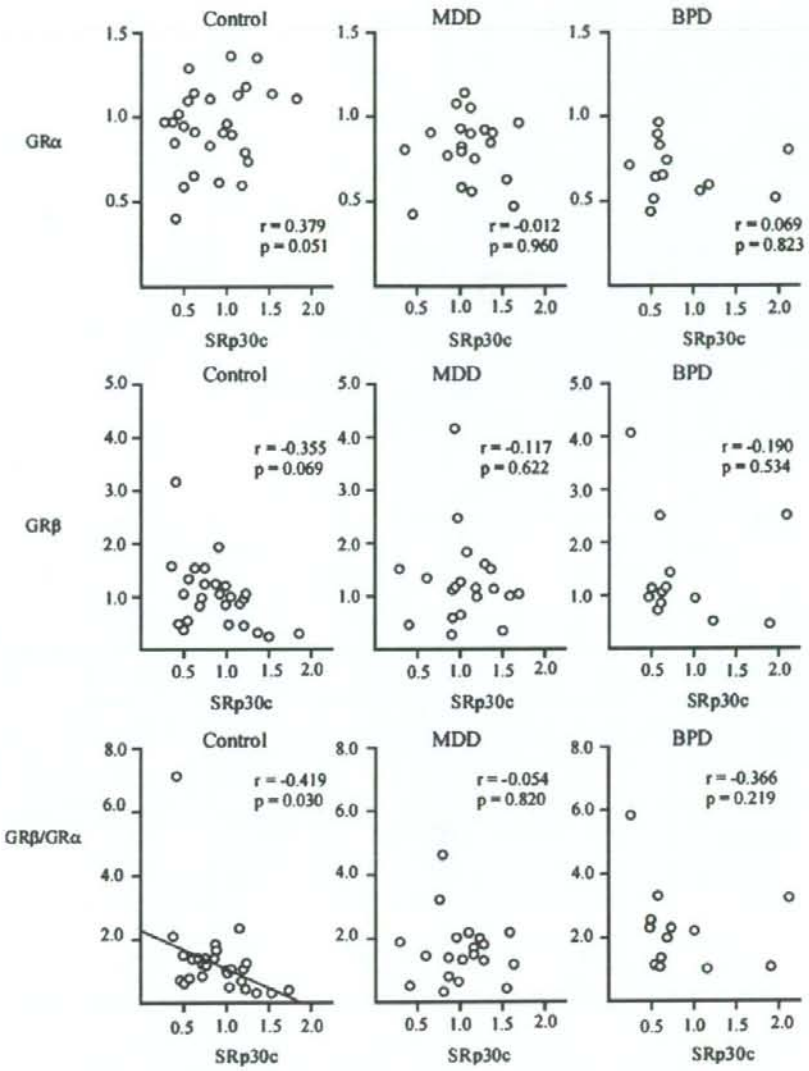

Fig. 2. The correlations of SRp30c mRNA expression with GRα mRNA, GRβ mRNA and the GRβ/GRα ratio. There was a significant correlation between SRp30c mRNA expression and the GRβ/GRα ratio only in healthy control subjects. Only data for patients in the depressive state are shown. MDD, major depressive disorder; BPD, bipolar disorder.

No such correlations were found in either BPD patients in a depressive state or healthy control subjects (Fig. 3). There were no significant correlations of SRp20 mRNA expression with serum cortisol concentration in healthy control subjects ($r=-0.030$, $p=0.906$), MDD patients ($r=0.285$, $p=0.458$), or BPD patients ($r=-0.066$, $p=0.916$) in the depressive state.

In the remissive state, SRp20 mRNA expression was not significantly correlated with GRα mRNA in MDD patients ($r=-0.009$, $p=0.954$) or BPD patients ($r=-0.107$, $p=0.529$). Also, there were no significant correlations between SRp20 mRNA expression and serum cortisol concentration in MDD patients ($r=0.254$, $p=0.382$) or BPD patients ($r=0.225$, $p=0.420$) in the remissive state. GRβ mRNA expression in mood disorder patients in the remissive state was not analyzed in our previous report, because it was not altered in such patients in the depressive state (Matsubara et al., 2006).

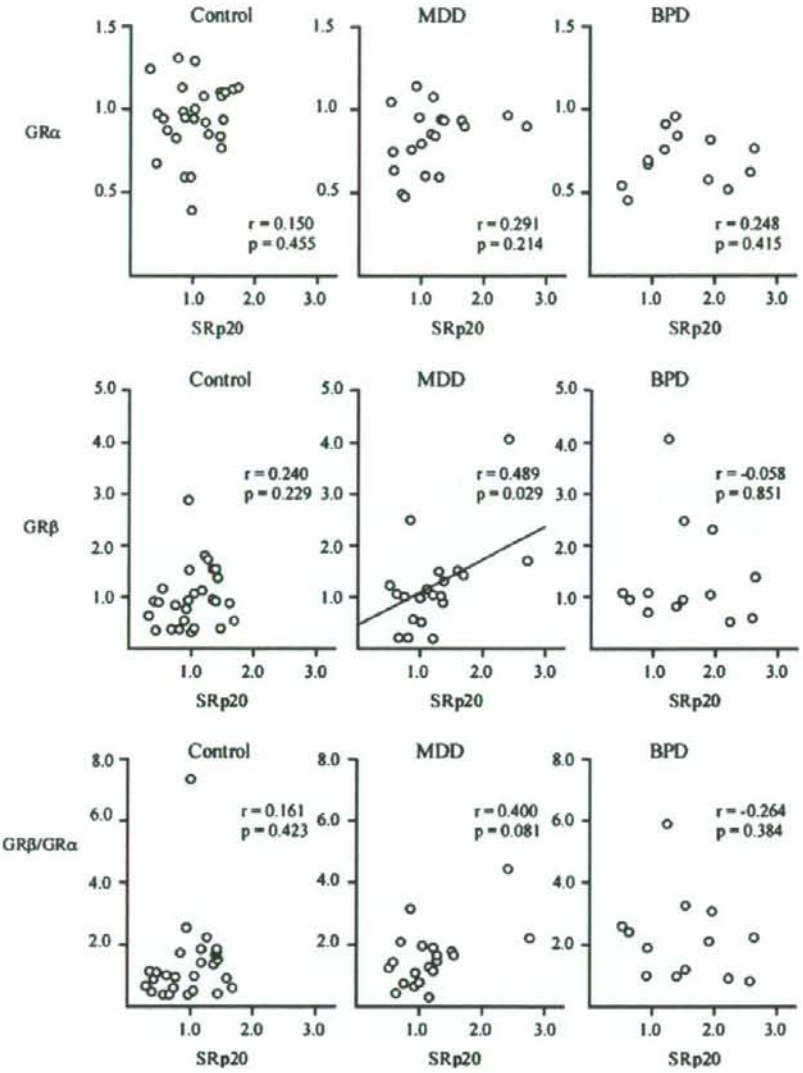

Fig. 3. The correlations of SRp20 mRNA expression with GRα mRNA, GRβ mRNA and GRβ/GRα ratio. A significant correlation of SRp20 mRNA expression with GRβ mRNA level was seen in depressive MDD patients, but not in healthy control subjects or in depressive BPD patients. Only data for patients in the depressive state are shown. MDD, major depressive disorder; BPD, bipolar disorder.

3.3. No significant relationship between SRp20 mRNA expression and medication

Recently, it was reported that valproic acid (VPA) increased SRp20 and ASF/SF2 protein levels (Brichta et al., 2003). To examine the effect of VPA on SRp20 mRNA expression in BPD patients, we divided them into two groups, those with or without VPA medication. The two groups showed similar increased SRp20 mRNA expression in both the depressive ($t=1.058$, df=11, $p=0.313$) and remissive ($t=-0.532$, df=35, $p=0.598$) states. To examine the effect of antidepressant medication on SRp20 mRNA expression in BPD patients, we studied the correlation between SRp20 mRNA expression and the dose of antidepressant medications; however, there were no significant correlations in patients in either the depressive ($r=-0.095$, $p=0.757$) or remissive ($r=0.025$, $p=0.884$) states.

4. Discussion

In the present study, we found the inverse correlation between SRp30c mRNA expression and the GRβ/GRα mRNA ratio in healthy control subjects, but not in mood disorder patients. This is the first evidence, *in vivo*, of the possibility that SRp30c might regulate the alternative splicing of GR, a finding that was reported previously only *in vitro* (Xu et al., 2003). Also, this finding supports our hypothesis that proper alternative splicing of the GR transcript may be disturbed in mood disorder patients, which is derived from our previous finding that a significant correlation between GRα and GRβ mRNA expression was found only in healthy control subjects, but not in mood disorder patients (Matsubara et al., 2006). Interestingly, there was also a significant correlation between SRp20 mRNA expression and GRβ mRNA expression in MDD patients, but not in BPD patients. This may be another finding suggesting that the alternative splicing status in mood disorder patients is different from that in the healthy controls. Although the present study revealed that there are altered correlative relationships between the expression of SR proteins and GR-related parameters in mood disorder patients, it remains unknown whether there are any causal relationships between them.

Another major finding of our study is that the expression of SRp20 mRNA was significantly increased in BPD patients, but not in MDD patients. The increased expression of SRp20 mRNA was revealed in these patients in both the depressive and remissive states. These findings suggest that increased expression of SRp20 mRNA in BPD patients may not be state-dependent. However, we did not identify significant alterations in SRp20 mRNA expression in the first-degree relatives of mood disorder patients. The increased SRp20 mRNA expression may be closely related to the onset of disease and last after recovery from the depressive state.

It has been reported that VPA increased SRp20 and ASF/SF2 protein levels in fibroblast cultures derived from spinal muscular atrophy patients (Brichta et al., 2003). However, we did not find such effects of VPA on SRp20 mRNA in the peripheral white blood cells of mood disorder patients, or ASF/SF2 mRNA expressions (data not shown). Thus, our findings did not suggest that the increased expression of SRp20 mRNA in BPD patients was caused by VPA medication. The discrepancy between the previous report and this study may be due to differences in the tissues examined.

The biological significance of increased SRp20 expression in BPD patients is the next question to be clarified. There are several target genes regulated by SRp20-mediated alternative splicing, such as calcitonin (CT)/calcitonin gene-related peptide (CGRP) and CD44 (Lou et al., 1998; Galiana-Arnoux et al., 2003). These molecules have been reported to be involved in the regulation of proinflammatory cytokines and the HPA axis (Noble et al., 1993; Dhillo et al., 2003), which are related to the pathophysiology of mood disorders.

Although it is unknown whether SRp20 expression is also changed in the brain of BPD patients, it should be noted that there have been an increasing number of molecules that showed altered expression in both brain and peripheral blood cells of mood disorder patients, including GRα mRNA, heat shock protein 40, and LIM protein (Webster et al., 2002; Iwamoto et al., 2004; Knable et al., 2004; Matsubara et al., 2006). To confirm the relevance of an altered SRp20 expression to the pathophysiology of mood disorders, it is necessary to establish an animal model for mood disorders, in which the SRp20 gene and/or its target genes are genetically modified.

This is the first report demonstrating the possibility of an aberrant alternative splicing occurring via increased SRp20 in BPD patients. The increased expression of SRp20 mRNA in BPD patients was consistent in both the depressive and remissive states. Our results suggest that SRp20 might also be a biological marker of BPD, by distinguishing it from MDD even at the first depression episode, and leading to appropriate psychopharmacological strategies for prevention of manic episodes.

Conflict of interest

There are no conflicts of interest including any financial, personal, or other relationships with people for any of the coauthors related to the work described in the article.

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