PROCEEDING

Assessment of human stress and depression by DNA microarray analysis

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Abstract : Precise assessment of stress is an imminent issue to deal with stress-related social, medical and psychological problems. Psychological stress is known to stimulate the neuroendocrine, sympathetic nervous, and immune systems. By analyzing mRNA expression levels in leukocytes, which express receptors for hormones, neurotransmitters, growth factors, cytokines, and other stress related signals, levels of stress may be adequately measured. In a series of studies, our group has developed a cDNA microarray specifically designed to measure the mRNA levels of stress-related genes in peripheral blood leukocytes. This microarray enabled us to sensitively detect the response to psychological stress. In addition, our preliminary study suggests that the array could differentiate patients with depression from sex- and age-matched control subjects. J. Med. Invest. 52 Suppl. : 266-271, November, 2005

Keywords : *stress, depression, biological maker, DNA microarray*

OBJECTIVE MEASUREMENT OF STRESS

Psychological stress influences levels of quality of life. Moreover, stress has been implicated in the pathogenesis of various psychiatric and psychosomatic disorders. Precise assessment of stress is an imminent issue to deal with stress-related social, medical and psychological problems. While most studies utilize subjective questionnaires for the assessment of psychological stress, some objective methods has been introduced. Psychological stress is known to stimulate the hypothalamus-pituitary-adrenal (HPA) axis, sympathetic nervous system, and immune system. These systems interact with each other, leading to the complex stress response (1, 2). In addition to corticotrophin-releasing hormone, adrenocorticotropic hormone, and glucocorticoids, physiological stress stimulates production of cytokines and modifies inflammatory and immune responses. Measurement of one of these hormones or cytokines has been used to objectively assess the levels of stress. However, their usefulness as a biological marker is limited, because of the unsatisfactory sensitivity and/or specificity.

One of the new approaches for the assessment of stress response is to use DNA microarray. The microarray is an emerging technology that allows simultaneous measurement of thousands of mRNA transcripts in biologic samples. The microarray is now recognized as a useful clinical device to make diagnostic, therapeutic, or prognostic decisions for patients. Considerable progress has already been made in clinical cancer researches, using systematic analysis of gene expression patterns to define tumor subtypes, identify molecular markers, and investigate new therapies (3-10). The examples of special note are applications in the differential diagnosis of adult acute leukemias (4) and the identification of clinical-outcome

Received for publication September 9, 2005 ; accepted September 16, 2005.

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predictors in adult acute myeloid leukemia (5) and breast cancer (6, 7). In addition to these applications, high-throughput analysis of gene expression by microarray may have a potential advantage of being able to study complex responses, such as psychological stress response, in which the measurement of limited numbers of gene products does not always reflect the status.

Peripheral leukocytes produce various cytokines, and proinflammatory cytokines, particularly gp130 family members, directly stimulate the HPA axis (11). At the same time, leukocytes express receptors for stress mediators, such as hormones, neurotransmitters, growth factors, and cytokines. Thus, leukocytes may be a potential target for the evaluation of psychological stress response. In a series of studies, we have developed a cDNA array specifically designed to measure the mRNA levels of stress-related genes in peripheral blood leukocytes (12).

DEVELOPMENT OF STRESS-SENSITIVE MICROARRAY

Construction of stress microarray and data analysis

We started to list stress-related genes (stress hormones, neurotransmitters, cytokines, growth factors, receptors, signal transduction molecules, transcription factors, heat shock proteins, growth- or apoptosis-associated factors, and metabolic enzymes) from the UniGene database of the National Center for Biotechnology Information (Ftp://ncbi.nlm.nih.gov.repository/unigene/). Target sequences of the listed genes were designed using original software (Hitachi, Saitama, Japan), and we selected 1,467 genes that were actually amplified by reverse transcriptase-PCR using total RNA isolated from peripheral leukocytes of healthy volunteers (see http://www.hitachi.co.jp/LS/ for the full list of genes). All PCR products were sequenced to be the corresponding cDNAs, and they were spotted on the array according to the method previously described (13). The microarray showed high reproducibility with a mean coefficient of variation of less than 20 %, and the dynamic ranges were three orders of magnitude.

Signal intensities of Cy5 and Cy3 were quantified and analyzed by subtracting the backgrounds, using QuantArray software (GSI-Lumonics). The intensity values for duplicate cDNA probes were averaged. Following global normalization, we selected several hundreds genes with a fluorescence intensity higher than the cut-off value of 300 in both conditions (labeled with Cy5 or Cy3). The relative expression values (Cy5/Cy3) for these genes were subjected to hierarchical clustering using GeneSpring 6.0 software (Silicon Genetics, Redwood City, CA, USA) and similarity analysis by standard correlation. After Cy5/Cy3 ratios of these genes were transformed to logarithms, data were statistically analyzed.

Effect of leukocyte preparation on gene expression profiles

Freshly isolated or cultured mononuclear cells are usually used to examine target genes relevant to disease pathogenesis (14). These preparations are also applied to expression analysis with microarray. It recently became possible to directly prepare RNA from whole blood using a commercially available kit, which may eliminate non-specific changes in mRNA levels during preparations (15, 16). When mRNA levels in isolated leukocytes (mixture of mononuclear cell- and neutrophil-rich fractions) were compared with those prepared from whole blood, the isolation procedures increased the expression values of 52 genes > 2-folds and decreased those for 10 genes to < 50 %. Thus, the isolation procedures, such as mechanical stimuli, significantly altered expression of stress-responsive genes. Whitney *et al.* also suggested that excessive *in vitro* handling required for isolation of monocytes from peripheral blood leads to a gene expression "signature" of cell stress, including up-regulation of v-fos (*FOS*), *CD83* and *CD69*, tumor necrosis factor (TNF)-α induced protein 3 (*TNFAIP3*), *DUSP2* (17). Based on this information, together with our findings, RNA samples directly prepared from whole blood were used to correctly assess the stress response in the following experiments.

Effects of exercise and daily activity

We examined how physical exercise affected the gene expression in peripheral leukocytes. Treadmill exercises under aerobic conditions (< 60% of VO_2 max for 1 h) did not change the expression pattern in 5 healthy volunteers, although exhaustive exercise with treadmill significantly changed it. We identified 26 genes whose expressions were significantly changed after the exhaustive exercise.

We also examined whether routine works affected the gene expression in peripheral leukocytes in daily life. It was suggested that lunch or dinner transiently changed the mRNA levels of 11 genes, such as IL-2 receptor β chain (*IL2RB*), *MYB*-related gene *BMYB* (*MYBL2*), IL-7 receptor (*IL7R*), general transcription factor IIF (*GTF2F1*), interferon inducible mRNA

fragment (*G1P3*), telomerase reverse transcriptase (*TERT*), phosphoinositide 3-kinase-associated p85 (*PIK3R1*), T-cell specific protein (*RANTES*), CDC-like kinase (*CLK1*), dihydropyrimidine dehydrogenase (*DPYD*), KIAA0822 protein (*ABCA8*). But other daily activities had no effect. In contrast, we confirmed that mRNA levels for the selected 70 genes were stable in daily life ; diet, classworks, or light exercises did not significantly alter their mRNA levels.

Assessment of stress with the stress-sensitive microarray

We asked graduate students who would take the final examination for the PhD to participate in this study. They were in good physical health, were taking no medication, and had no history of psychiatric or somatic diseases. The final examination consisted of an oral presentation of the PhD theses and a question-and-answer session. Venous blood (10 ml) was taken from each subject 2 h before and 2 or 24 h after the examination. The sample collected 4 wk before the presentation was used as a reference. All blood samples were collected under fasting conditions.

Hierarchical cluster analysis of the relative expression values identified two groups of genes, whose expressions were uniformly up-regulated or down-regulated at 2 h after the presentation. Most of the expression levels returned to the baseline within 24 h after the examination. Bayesian *t*-test (error rate = 0.05) identified 70 genes whose mRNA levels were significantly changed at 2 h after the examination.

The neuroendocrine response, activated by psychological stress, converts stress into changes in mononuclear cell functions (18), and stimulates the production of TNF-α, IL-6, IL-1 receptor antagonist, interferon (IFN)-γ, and IL-10 (19). The significantly up-regulated genes included receptors for these cytokines and their associated molecules : IL-1 receptor (*IL1R1* and *IL1R2*), TNF receptor homologue (*TNFRSF10C*), TNF-α-induced protein (*TNFAIP6*), IFN-γ receptor 2 (*IFNGR2*), interferon (*IFN*)-induced cellular resistance mediator protein (*MX2*), IFN-regulatory factor-2 (*IRF2*), and IFN-inducible proteins (*IFITM1* and *IFITM3*). This suggests that the stress-responsive cytokines may stimulate their receptor signals in peripheral leukocytes in response to the stress. In addition, the stressful event significantly up-regulated the expression values of several other cytokine/chemokine receptors and their related genes, such as colony-stimulating factor receptors (*CSF2R* and *CSF3*), *IL17R*, *IL8RA*, *IL8RB*, chemokine ligands (*SCYB5* and *GRO1*), Fc fragment of IgG (*FCGR2B*), IL-regulated nuclear factor (*NFIL3*), and selectin L (*SELL*).

The mRNAs for stress-mediating molecules including hypoxia-inducible factor 1 (*HIF1A*), *FOS*, and p38 MAP kinase (*MAPK14*), as well as those for heat shock protein 70 members (*HSPA6* and *HSPA1A*), were also significantly up-regulated. The activation of catecholamine receptors and glucocorticoid receptor indirectly or directly modifies the transcription of various genes (20, 21). Psychological stress rapidly and transiently activates nuclear factor-κB, a hallmark of inflammatory responses (22), in association with elevated levels of catecholamines and cortisol (18). Thus, psychological stress activates multiple signaling pathways ; therefore it is difficult to fully explain the biological significance of several other genes. With regard to the significantly down-regulated genes, however, the life event stress generally down-regulated mRNA expression for growth-related genes and cytochrome *c* oxidase subunits.

BIOLOGICAL MARKERS OF DEPRESSION

Depression affects about 10 % of the population at some point in their life and is the leading cause of disability in the nations with developed economies (23). The disease is potentially fatal because 15% of patients with severe depression eventually die by suicide (24). If treated properly, most patients would recover from the disease. However, studies have shown that depression, which lacks specific objective findings, is often missed or undiagnosed (25). Establishment of convenient and reliable biological markers would greatly improve the precise diagnosis and consequently the welfare of depressed patients.

Depression affects not only the mind but also the entire organ systems through the interaction between the brain and the body. There are convincing evidences indicating endocrinological, immunological and autonomic nervous system disturbances in depression (26). A large number of studies have been conducted to establish a diagnostic marker by detecting one of these systemic disturbances. One of the examples most extensively studied is the attempt to detect the hyperactivity of HPA axis by using DST or its modification such as Dex/CRH test (27). Measurement of neurotransmitter receptors or transporters located on the blood cells have been also studied vigorously on the assumption that they reflect to some extent their counterparts in the CNS. For example, decreased 5HTT binding have been reported in platelets of depressive patients (28, 29), although some studies reported no change

(30-32). More recently, with the progress of experimental procedure, altered mRNA levels in leukocytes have been reported, such as decreased dopamine D4 receptor mRNA levels (33) or increased serotonin transporter mRNA levels (34) in major depression and decreased CREB mRNA levels in treated major depression (35). Our group has found increased serotonin transporter mRNA levels in leukocytes. Although these and other studies with peripheral measures have contributed greatly to the understanding of the mood disorders, its usefulness as a biological marker in the clinical setting is limited, because of the unsatisfactory sensitivity and/or specificity as well as the troublesome procedure requiring the intense cooperation of patients.

ASSESSMENT OF DEPRESSION WITH THE STRESS-SENSITIVE MICROARRAY

We have been exploring to establish a new biological marker of depression by profiling mRNA expression from the leukocytes of the patients by using the stress sensitive microarray. As is the case with psychological stress, we predict that the characteristic abnormalities in the neuroendocrine, neuroimmune and autonomic nervous system in depression should have effects on the expressional pattern of mRNA in the leukocytes from depressed patients.

We have reported preliminary results with 32 patients (36). They met the criteria of Major Depressive Disorder according to Diagnostic and Statistical Manual of Mental Disorders, Fourth Edition (37), aged from 18 to 65, with no psychiatric comorbidities, no serious somatic diseases, and no use of antidepressants. Hamilton Rating Scale for Depression (HAM-D) and Clinical Global Impression Scale (CGI) were used for clinical evaluation. Peripheral blood was collected before the treatment and expression levels were compared with sex-and age-matched healthy volunteers.

The microaray analysis revealed that expression of a dozen of genes showed significant changes in the total group of the patients compared with controls. These genes may be useful as biological makers of depression. In addition to these genes, there were dozens of genes significantly altered in a half of the patients compared with controls, thus dividing the patients into two groups, although no symptomatic or demographic variables could account for the distinction. Altered expression of genes of various categories in a subgroup of depression might be relevant to the pathophysiology of this subgroup. It was also of note that some of the altered genes before treatment significantly changed toward the reverse direction after treatment.

These preliminary results revealed sets of gene expressions that could distinguish depressed patients from controls. These alterations were different from those observed in volunteers after stress or those in preliminary samples of patients with schizophrenia. Mechanisms of the alteration remain unclear. Neurotransmitter, endocrinological and immunological abnormalities are thought to have contributed to the alteration of the expression to some extent. Some of the alteration may directly reflect intracellular abnormalities of depression that might be present in the leukocytes. These altered genes provide clues for elucidating molecular mechanisms of the disease. The microarray method has a great advantage over pervious biological markers in that it can utilize hundreds of parameters from a small amount of peripheral blood cells. Thus, a complicated and probably heterogeneous disease such as depression could be adequately recognized. This approach has opened a novel and promising horizon in the search of a biological marker of depression.

CONCLUSION

Adequate biological assessment of stress would help to solve stress related social, medical and psychological problems. High-throughput analysis of gene expression by microarray may have a potential advantage of being able to study complex responses, such as psychological stress response, in which the measurement of limited numbers of gene products does not always reflect the status. We developed a cDNA microarray specifically designed to measure the mRNA levels of stress-related genes in peripheral blood leukocytes. Using this microarray, mRNA expressional change under stress was detected with good sensitivity and specificity. Moreover, preliminary results suggest that the array could differentiate patients with depression from sex-and age-matched control subjects. Thus, DNA microarray with stress-related genes may provide a new biological maker for stress response and stress-related disorders.

ACKNOWLEDGEMENT

This work was supported by a Grant-in-Aid for

Scientific Research and the Special Coordination Funds for Promoting Science and Technology from the Ministry of Education, Culture, Sports, Science and Technology (KR), a Health and Labor Science Research Grant from the Ministry of Health, Labor and welfare (TO), and a Grants-in-Aid for Scientific Research from the Ministry of Education, Culture, Sports, Science and Technology and a Grants-in-Aid for Scientific Research from the 21st Century COE Program, Human Nutritional Science on Stress Control Tokushima, Japan.

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Neuroscience Letters 397 (2006) 25–29
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The Val66Met polymorphism of the brain-derived neurotrophic factor gene affects age-related brain morphology

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Received 18 October 2005; received in revised form 22 November 2005; accepted 30 November 2005

Abstract

We investigated the effects of the brain-derived neurotrophic factor (BDNF) Val66Met polymorphism on age-associated changes of brain morphology in 109 Japanese healthy subjects using MRI with optimized voxel-based morphometry technique. A significant age-related volume reduction was found in the dorsolateral prefrontal cortices (DLPFC), anterior cingulate cortices, and temporal and parietal cortices in all subjects. Further analysis revealed a significantly negative correlation between age and the volume of the bilateral DLPFC only in the Met-BDNF carriers, and a significant interaction between the polymorphism and age-associated volume changes in the bilateral DLPFC. Furthermore, Met-carriers showed a significant interaction ($p < 0.0001$) between the gender and the genotype on the gray matter volume in the DLPFC, and female Met-carriers showed more widespread age-associated volume reduction in DLPFC than male Met-carriers. Our data suggest that the Val66Met polymorphism may impact on age-related changes of the brain, which might be associated with the functional variance of neuroprotective effects of the BDNF. Furthermore, we suggest that genotype effects of the BDNF gene on brain morphology might differ in female from in male.

Keywords: Brain-derived neurotrophic factor; Val66Met polymorphism; Magnetic resonance imaging; Voxel-based morphometry; Dorsolateral prefrontal cortex; Aging

Brain-derived neurotrophic factor (BDNF), a member of neurotrophin family, has important roles in hippocampal plasticity and hippocampal-related learning and memory through long-term potentiation [15]. It also plays an important role in preventing death of neurons during development and protecting cholinergic neurons of the basal forebrain and the hippocampus from induced death in the adult brain [21].

A common missense polymorphism of the BDNF gene producing a valine to methionine amino acid substitution (Val66Met) affects the activity dependent secretion of BDNF in neurons and affects memory function [6,8]. Neuroimaging studies revealed that this polymorphism affected memory-related neuronal activities measured by functional magnetic resonance imaging (MRI) and macroscopic morphology of the hippocampus [8,12,23,28]. Regarding the brain morphology in normal individuals, Pezawas et al. [23] reported that Met-BDNF carriers had smaller volumes of the hippocampi and the prefrontal cortices as compared to individuals with homozygous Val-BDNF. This result was recently replicated in another mixed study of healthy and schizophrenic subjects [28]. Although several neuroimaging studies have indicated that environmental factors considerably impact on human brain structures even in normal adult brains [18], these data suggest that genetic factors such as polymorphism of BDNF might also strongly affect human brain morphology, and contribute to individual differences of brain morphology.

Aging is another factor which strongly affects brain morphology in human. There are several studies that demon-

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doi:10.1016/j.neulet.2005.11.067

strated morphological changes associated with normal aging in vivo [10,24]. A general trend in the in vivo volumetric studies of healthy volunteers points to the prefrontal cortex as the cortical region in which the largest age-related volume reduction is observed. Considering the previous findings that BDNF is expressed abundantly in the prefrontal cortex [25] and that BDNF has a neuroprotective effect, Val66Met polymorphism might have some impacts on age-related morphological changes. However, there is no datum whether this polymorphism is associated with age-related morphological changes.

To clarify whether the BDNF polymorphism impacts on morphological changes associated with aging, we analyzed structural MR images in 109 normal individuals using optimized voxel-based morphometry (VBM) technique.

One hundred and thirty healthy subjects participated in the study. Written informed consent was obtained from all subjects in accord with ethical guidelines in place at local ethical committee. All of the subjects were recruited from local advertisements and underwent a Japanese version of National Adult Reading Test (JART) that is essentially the same as National Adult Reading Test [22] and MRI scanning. We employed JART as a convenient tool to measure IQ for each participant because previous study reported that it showed high correlation with IQ in healthy subjects [20]. All subjects were screened by a questionnaire regarding medical history and excluded if they had neurological, psychiatric or medical conditions that could potentially affect the central nervous system, such as substance abuse or dependence, atypical headache, head trauma with loss of consciousness, asymptomatic or symptomatic cerebral infarction detected by T2 weighted MRI, hypertension, chronic lung disease, kidney disease, chronic hepatic disease, cancer, or diabetes mellitus. Template creation for the optimized VBM was based on a sample of the 120 subjects, aged 36.2 ± 12.1 years (range 20–72). All subjects were Japanese. Since single nucleotide polymorphism (SNP) genotyping, described in the next section, was done successfully in 109 subjects, the MR images of these 109 subjects were used for subsequent analyses. According to the polymorphism, subjects were categorized into the following three groups: a homozygous Val-BDNF group ($n=41$), a Val/Met-BDNF group ($n=51$), or a homozygous Met-BDNF group ($n=17$). The genotype distribution of this SNP was not deviated with Hardy–Weinberg equilibrium (chi-square = 0.03, $p=0.86$). Because of the small number of subjects with homozygous Met-BDNF, the Val/Met-BDNF group and homozygous Met-BDNF group were treated as one group, the Met-BDNF carriers ($n=68$). The demographic data of these groups are the following; the homozygous Val-BDNF comprised 26 females and 15 males, two were left-handed, aged 36.9 ± 13.0 years (range 21–68), and the mean education period and JART score were 16.2 ± 2.8 years (range 12–24) and 75.5 ± 13.3 (equivalent to 108.8 ± 9.55 for full scale IQ (range 50–96; equivalent to 90.5–123.6 for full scale IQ), respectively. The Met-BDNF carriers comprised 45 females and 23 males, three were left-handed, aged 35.8 ± 11.6 years (range 20–72), and their mean education period and JART score were 16.9 ± 3.0 years (range 12–28) and 78.0 ± 11.6 (equivalent to 110.7 ± 8.3) for full scale IQ (range 45–99; equivalent to 86.9–125.8 for full scale IQ), respectively. The mean age, gender ratio, handedness, education period, or JART score did not differ between the two groups (two sample t-test, data not shown).

The detail process of genotyping of BDNF Val66Met SNP (dbSNP accession: rs6265) was described previously [13]. Primers and probes for detection of the SNP (TaqMan SNP Genotyping assays on demand) were purchased from Applied Biosystems (ABI, Foster City, CA, USA). PCR cycling conditions were: at 95 °C for 10 min, 50 cycles of 92 °C for 15 s and 60 °C for 1 min.

All MR studies were performed on a 1.5 T Siemens Magnetom Vision plus system. A three dimensional volumetric acquisition of a T1-weighted gradient echo sequence produced a gapless series of 144 sagittal sections using an MPRage sequence (TE/TR, 4.4/11.4 ms; flip angle, 15°; acquisition matrix, 256×256; 1NEX, field of view, 31.5 cm; slice thickness, 1.23 mm).

Data were analyzed with Statistical Parametric Mapping 2 (SPM2) (http://www.fil.ion.ucl.ac.uk/spm/; Wellcome Department of Imaging Neuroscience, London, UK) running on MATLAB 6.5 R1 (MathWorks, Natick, MA). Before analyses, each image was confirmed by a neuroradiologist to eliminate images with artifacts, and then anterior commissure–posterior commissure line was adjusted. First, we made a customized anatomical T1 template and prior probability images from the sample of 120 brains [10]. Then, images were processed using an optimized VBM script (dbm.neuro.uni-jena.de/vbm.html). The detail of this process is described elsewhere [2,10]. The normalized segmented images were modulated by multiplication with Jacobian determinants of the spatial normalization function to encode the deformation field for each subject as tissue density changes in the normal space. Finally, images were smoothed using a 12 mm full width half maximum of isotropic Gaussian kernel. Statistical analyses were performed with SPM2, which implemented a General Linear Model. Proportional scaling was used to achieve global normalization of voxel values between images. First, we used a two-sample t-test to test regional population effect on gray matter volume. For this analysis, we set $p<0.005$ without a correction for multiple comparisons, followed by applying small volume correction to each cluster with a false discovery rate (FDR) < 0.05. For the small volume correction, spheres with radius 10 mm around the peak were set as regions of interest (ROIs). The resulting sets of t-values constituted the statistical parametric maps {SPM (t)}. Anatomic localization was according to both MNI coordinates and Talairach coordinates, obtained from M. Brett's transformations (http://www.mrc-cbu.cam.ac.uk/Imaging/Common/mnispace.shtml) and presented as Talairach coordinates. Since a previous study with Caucasians demonstrated a significant reduction of volumes in the hippocampi and the frontal cortices in Met-BDNF carriers, we applied an additional hypothesis-driven ROI method to test regional population effects in these regions by using the Wake Forest University PickAtlas [19].

The genotype effects on age-related morphological changes were tested using a single subject condition and covariate model. Since several studies reported gender different age-related mor-

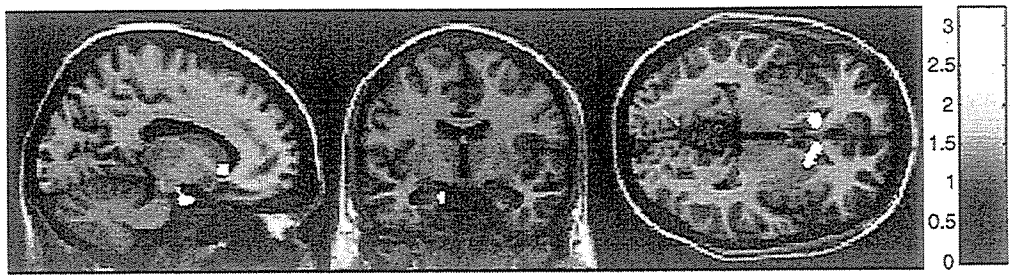

Fig. 1. The volume reduction of Met-BDNF carriers compared to that of individuals with homozygous Val-BDNF ($p < 0.05$, small volume correction with FDR). A significant reduction of volumes of the left parahippocampal gyrus (t-value: 2.92, Talairach coordinates (TAL): −12, −3, −19) and the bilateral heads of caudate nucleus (left: t-value; 3.23, TAL: −9, 22, −3, right: t-value; 3.02, TAL: 10, 21, −4) in the Met-BDNF carriers was noted.

phological changes in the brain [7], we additionally examined genotype effects on age-related morphological changes in each gender, separately. Orthogonalized first order polynomial expansion of age was treated as a covariate of interest to determine the linear effects of age [5]. Since second- and third-order polynomial expansions did not contribute to the age effect model of our sample, we removed them from a design matrix. Considering the possible association between IQ and brain morphology, we treated JART score as a nuisance variable. For this analysis, we applied $p < 0.001$, corrected for multiple comparisons with FDR < 0.05 as a statistical threshold [9]. MarsBar program (marsbar.sourceforge.net/) was also used to extract data from the regions of interest.

Fig. 1 shows a significant reduction of gray matter volumes of the left parahippocampal gyrus (Brodmann area (BA) 34), and bilateral heads of the caudate nucleus in Met-BDNF carriers when compared to homozygous Val-BDNF individuals. Even in hypothesis-driven ROI approach with a lenient statistical threshold (uncorrected $p = 0.05$), we could not find any significant differences of hippocampal nor prefrontal cortical volumes between the two groups. The results were essentially unchanged even when the restricted samples of subjects (female group, male group, or young group aged under 40 years old) were analyzed (data not shown).

Fig. 2 shows morphological changes related to normal aging. A significant negative correlation between age and the gray matter volumes was noted in the bilateral dorsolateral prefrontal cortices (DLPFC; BA9, 46), right superior temporal gyrus (STG; BA22), bilateral insulae (BA13), bilateral caudate nuclei, left anterior cingulate gyrus (BA24), bilateral inferior parietal lobules (BA40), bilateral precunei (BA7), and bilateral fusiform gyri (BA37) in all subjects. In homozygous Val-BDNF individuals, a significant age-related volume reduction was found in the bilateral insulae (BA13) and right STG (BA22). On the other hand, Met-BDNF carrier showed an additional negative correlation of the gray matter volumes in the bilateral DLPFC (BA9, 46) and right dorsal premotor area (BA6) with age. Additional analyses in each gender revealed a significant interaction ($p < 0.0001$) in Met-carriers between the gender and the genotype on the gray matter volume in the DLPFC, and female Met-carriers showed more widespread age-associated volume reduction in DLPFC than male Met-carriers. Male Met-carrier also showed volume

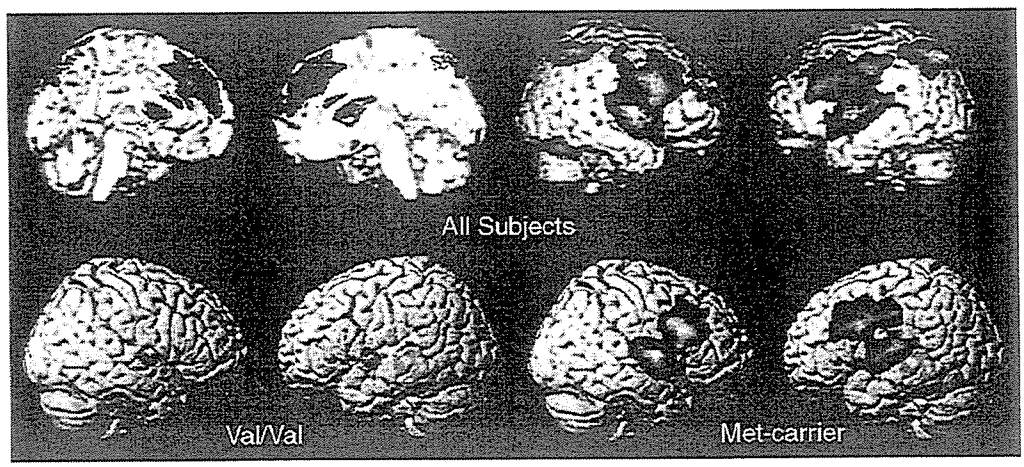

Fig. 2. (Top) The volume reduction associated with normal aging in all subjects ($p < 0.05$, FDR corrected). All subjects showed negative correlation with age in the bilateral DLPFC, right STG, bilateral insulae, bilateral caudate nuclei, left anterior cingulate gyrus, bilateral inferior parietal lobules, bilateral precunei, and bilateral fusiform gyri. (Bottom) The volume reduction associated with normal aging in each genotypic group ($p < 0.05$, FDR corrected). (Left) Results of individuals with homozygous Val-BDNF. Individuals with homozygous Val-BDNF showed negative correlation with age in the bilateral insulae (right: t-value: 4.36, TAL: 42, −2, 4; left: t-value: 4.52, TAL: −43, −2, 4) and the right superior temporal gyrus (t-value: 4.57, TAL: 47, 9, −4). (Right) Results of Met-BDNF carriers. The Met-BDNF carriers showed negative correlation with age in the bilateral dorsolateral prefrontal cortices (right: t-value: 6.5, TAL: 52, 21, 26; left: t-value: 6.12, TAL: −48, 19, 32) as well as the bilateral insulae and the superior temporal gyri.

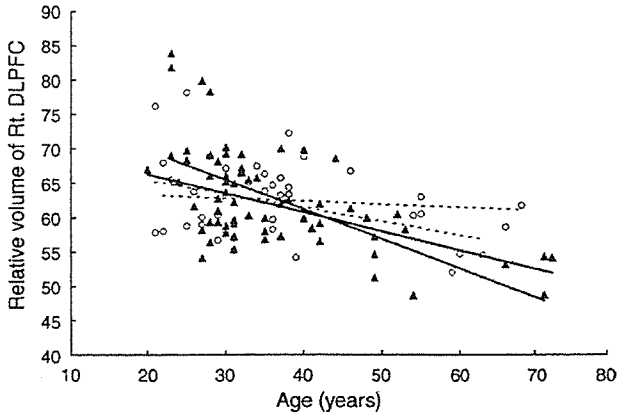

Fig. 3. Scatter plot of relative gray matter volume of the right DLPFC against age in each genomic group. The Met-BDNF carriers showed more significant volume reduction with normal aging compared to homozygous Val-BDNF subjects in the bilateral DLPFC in each gender (right: male Met-BDNF carriers: $y=-0.27x+71.8$, $r=-0.71$, $p<0.0001$, male homozygous Val-BDNF subjects: $y=-0.046x+64.2$, $r=-0.12$, $p=0.67$, female Met-BDNF carriers: $y=-0.43x+78.4$, $r=-0.56$, $p<0.001$, female homozygous Val-BDNF subjects: $y=-0.20x+69.5$, $r=-0.41$, $p=0.03$; left: male Met-BDNF carriers: $y=-0.20x+67.2$, $r=-0.53$, $p=0.01$, male homozygous Val-BDNF: $y=-0.11x+65.3$, $r=-0.25$, $p=0.367$, female Met-BDNF carriers: $y=-0.48x+77.0$, $r=-0.71$, $p<0.0001$, female homozygous Val-BDNF: $y=-0.14x+65.3$, $r=-0.27$, $p=0.18$). Due to limitations of space, only the plot at the right DLPFC in each gender is shown. Blue stands for male subjects and red stands for female subjects. Open circle: homozygous Val-BDNF; closed triangle: Met-BDNF carrier. Dotted lines are the regression line of homozygous Val-BDNF, whereas solid lines are those of Met-BDNF carrier.

reduction in the right inferior parietal lobules (BA40, t-value: 3.86, Talairach coordinates: 40, −43, 53). We found a significant interaction effect (male: $p=0.003$, female: $p<0.0001$) between the aging effect and the genotype on the gray matter volume in the DLPFC in each gender. (right: male Met-BDNF carriers: $r=-0.71, p<0.001$, male homozygous Val-BDNF subjects: $r=-0.12, p=0.67$; female Met-BDNF carriers: $r=-0.56$, $p<0.001$, female homozygous Val-BDNF subjects: $r=-0.41$, $p=0.03$; left: male Met-BDNF carriers: $r=-0.53$, $p=0.01$, male homozygous Val-BDNF subjects: $r=-0.25$, $p=0.367$, female Met-BDNF carriers: $r=-0.71$, $p<0.0001$, female homozygous Val-BDNF subjects: $r=-0.27, p=0.18$) (Fig. 3).

This is the first study which investigated the impacts of BDNF Val66Met polymorphism on age-associated brain morphological changes in normal individuals. We found an exaggerated age-related volume reduction of the DLPFC in the Met-BDNF carriers.

Several studies demonstrated morphological changes associated with normal aging in the STG, insula, inferior parietal lobules, motor cortex, ACC, and DLPFC [10,24]. In consistent with previous studies, our data also showed age-related volume reduction in similar regions in all subjects' analysis of each gender. Further analysis revealed that the Met-BDNF carriers showed a stronger negative correlation between age and gray matter volume in the DLPFC and right precentral gyrus when compared to individuals with homozygous Val-BDNF. Though the mechanisms underlying the predilection of the prefrontal cortex for age-related volume reduction are still unclear, the prefrontal cortex exhibits the greatest age-related alteration of GABA and glutamate [11], and glucose metabolism and age-related declines in regional cerebral blood flow [4]. Though there has been no study investigating the relationship between Val66Met SNP and vulnerability to age-related changes, BDNF protein itself is reported to be associated with aging. Amounts of BDNF protein in hippocampal pyramidal neurons and dentate granule cells are decreased during aging in monkeys [14]. Further, several studies demonstrated neuroprotective effects of BDNF [3,29]. Our data suggest that the Met-BDNF carriers, particularly females carrying Met-BDNF allele, may be more vulnerable to aging than individuals with homozygous Val-BDNF. Considering the fact that prefrontal cortex is one of the regions in which BDNF is expressed abundantly [25], we suggest that the Val66Met polymorphism may be associated with functional variances of neuroprotective and stress resistant effects of BDNF, which results in different effects on age-related morphological changes. Furthermore, we found a reduction of the striatal volumes in met-BDNF carriers as compared to individuals with homozygous Val-BDNF. It has been postulated that enhancement of BDNF in the cortex may be involved in protection of striatal neurons against damage via anterograde transport because BDNF exerts neuroprotective effects against excitotoxicity in the striatum [1,16]. The result, reduced volumes in the striatum in met-BDNF carriers, may again suggest the reduced neuroprotective effects of met-BDNF. Since there has been no direct evidence of differential regulation of vulnerability to neurodegenerative process by BDNF Val66Met polymorphism, further study such as investigating how Val66Met SNP affects cell survival in a cellular model is required to clarify our speculation.

Although we could not replicate results of the previous studies, the smaller hippocampus in the Met-BDNF carriers [23,28], our data also suggest that BDNF polymorphism should have impacts on brain morphology associated with episodic memory. The discrepancy between our results and those of the previous studies could be partially explained by the racial difference. Binding its receptor TrkB, BDNF activates several pathways including the PI3-kinase/Akt, the mitogen-activated protein kinase, and PLC-gamma1 pathway [15]. These signals are known to be critical for survival of neuron, suggesting that not only Val66Met polymorphism of BDNF, but also interaction of polymorphism of each signal or molecule has effects on brain morphology. Racial differences might be related to such interactions, resulting in the different findings. This may partially contribute to the discrepancy in associations between BDNF polymorphism and the prevalence of neuropsychiatric diseases in Asian and Caucasian populations [17,27].

Finally, we mention a limitation of this study. To explore the association between aging effects on the brain morphology and the Val66Met polymorphism, we performed a cross-sectional study. There is a secular bias, which can be resolved by a longitudinal study. In this context, our data may be considered preliminary rather than conclusive. However, a recent longitudinal MR study of normal aging demonstrated that cross-sectional and longitudinal estimates of atrophy rates were similar [26].

In conclusion, we found that Val66Met polymorphism of BDNF had impacts on age-associated morphological changes in Japanese subjects. Our data suggest that Val66Met polymorphism of BDNF may play important roles for vulnerability to age-related morphological changes as well as the efficiency of plasticity, especially in DLPFC. Furthermore, we suggest that genotype effects of the BDNF gene on brain morphology might differ in female from in male.

Acknowledgements

The authors thank Ms. Tomoko Shizuno and Ms. Keiko Okada for technical assistance. This work was supported in part by Grants-in-Aid from the Japanese Ministry of Health, Labor and Welfare (H17-kokoro-007 and H16-kokoro-002), the Japanese Ministry of Education, Culture, Sports, Science and Technology, Core research for Evolutional Science and Technology (CREST) of Japan Science and Technology Agency (JST), Japan Foundation for Neuroscience and Mental Health, and the Program for Promotion of Fundamental Studies in Health Science of the Organization for Pharmaceuticals and Medical Devices Agency (PMDA).

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The Breakpoint Cluster Region Gene on Chromosome 22q11 Is Associated with Bipolar Disorder

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Background: *Although the pathogenesis of bipolar disorder remains unclear, heritable factors have been shown to be involved. The breakpoint cluster region (BCR) gene is located on chromosome 22q11, one of the most significant susceptibility loci in bipolar disorder linkage studies. The BCR gene encodes a Rho GTPase activating protein, which is known to play important roles in neurite growth and axonal guidance.*

Methods: *We examined patients with bipolar disorder (n = 171), major depressive disorder (n = 329) and controls (n = 351) in Japanese ethnicity for genetic association using eleven single nucleotide polymorphisms (SNPs), including a missense one (A2387G; N796S), in the genomic region of BCR.*

Results: *Significant allelic associations with bipolar disorder were observed for three SNPs, and associations with bipolar II disorder were observed in ten SNPs including N796S SNP (bipolar disorder, p = .0054; bipolar II disorder p = .0014). There was a significant association with major depression in six SNPs. S796 allele carriers were in excess in bipolar II patients (p = .0046, odds ratio = 3.1, 95% CI 1.53–8.76). Furthermore, we found a stronger evidence for association with bipolar II disorder in a multi-marker haplotype analysis (p = .0002).*

Conclusions: *Our results suggest that genetic variations in the BCR gene could confer susceptibility to bipolar disorder and major depressive disorder.*

Key Words: Breakpoint cluster region (BCR), bipolar disorder, major depression, 22q, association study, single nucleotide polymorphism (SNP)

Bipolar disorder is a major psychiatric disorder that is characterized by fluctuation between abnormal mood states of mania and depression. Since lithium has been one of the primary drugs used to treat bipolar disorder, molecular and cellular actions of this drug are believed to be clues of the pathophysiology of this disease, e.g. inhibition of glycogen synthase kinase-3, inositol monophosphatase, or N-methyl-D-aspartate (NMDA) receptor activity, activation of BDNF/Trk pathway, and enhancement of neurogenesis and neuronal progenitor proliferation (Chen et al 2000; Hallcher and Sherman 1980; Hashimoto et al 2002a, 2002b, 2003; Klein and Melton 1996). Recently, a common mechanism of action for three mood-stabilizing drugs, lithium, valproate and carbamazepine, has been identified (Williams et al 2002). These drugs inhibit the collapse of sensory neuron growth cones and increase growth cone area. Inositol reverses the effects of the drugs on growth cones, implicating inositol depletion in their action.

The breakpoint cluster region (BCR) gene is located on chromosome 22q, one of the most consistently replicated susceptibility loci in linkage studies of bipolar disorder (Detera-Wadleigh et al 1999; Edenberg et al 1997; Kelsoe et al 2001; Turecki et al 2001). A recent meta-analysis of eleven published genome scans for bipolar disorder revealed the strongest evidence for susceptibility loci on 22q and 13q (Badner and Gershon 2002). The BCR gene encodes a Rho GTPase-activating protein (GAP) highly expressed in hippocampal pyramidal cell layer and dentate gyrus (Fioretos et al 1995). The Rho family of GTP binding proteins acts as a key regulator for developing neuronal network, e.g. growth cone and neurite formation (Negishi and Katoh 2002). These proteins cycle between active GTP-bound and inactive GDP-bound forms. The activation of GTP-bound form is regulated by GAPs, which stimulate GTP hydrolysis, leading to inactivation (Etienne-Manneville and Hall 2002).

Therefore, genetic variability of the BCR gene is of considerable interest in the evaluation of risk of bipolar disorder. To our knowledge, however, there is no study examining the possible association between the BCR gene and bipolar disorder. The BCR gene (Online Mendelian Inheritance in Man [OMIM]:151410) consists of 23 exons and 22 introns, spanning 135 Kb. We searched for polymorphisms in the BCR gene in silico and selected eleven single nucleotide polymorphisms (SNPs), including a common single nucleotide substitution (A2387G; National Center for Biotechnology Information [NCBI] SNP ID: rs140504) in exon 10 giving rise to an amino acid change of asparagine to serine at codon 796 (N796S; NCBI Protein ID: NP_004318). In the present study, we performed an association study with SNPs in the region of the BCR gene in a Japanese population of bipolar and major depression cases and controls.

Methods and Materials

Subjects

Subjects were 171 patients with bipolar disorder (65 males and 106 females with mean age of 50.8 years [SD 14.9] and mean age of onset of 39.2 years [SD 15.2], 102 bipolar I [43 males and 59 females] and 69 bipolar II patients [22 males and 47 females]), 329 patients with major depressive disorder (116 males and 213 females with mean age of 54.3 years [SD 16.0] and mean age of onset of 46.7 years [SD 15.3]) and 351 healthy controls (170 males

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Received August 5, 2004; revised February 2, 2005; accepted February 14, 2005.

0006-3223/05/$30.00
doi:10.1016/j.biopsych.2005.02.019

BIOL PSYCHIATRY 2005;57:1097–1102

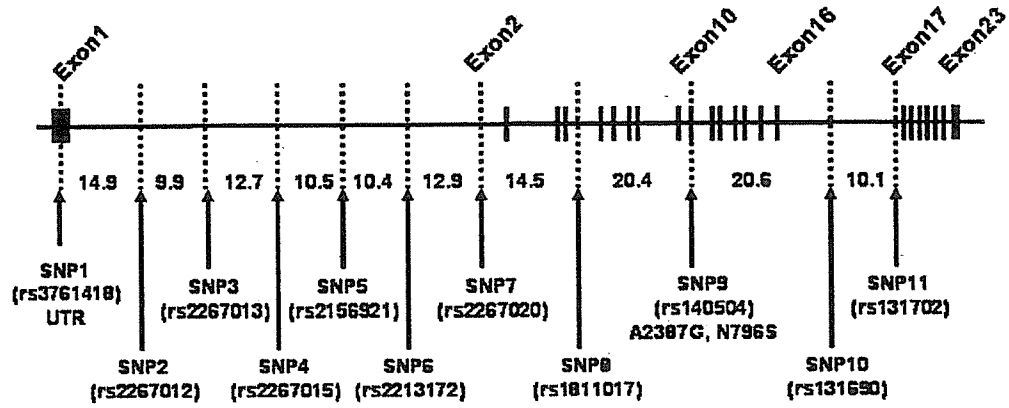

Figure 1. Genomic structure and location of single nucleotide polymorphisms (SNPs) for human Breakpoint Cluster Region (*BCR*) gene. Exons are denoted by bold vertical lines in black. The rs number of each SNP is the National Center for Biotechnology Information SNP cluster ID from the dbSNP database. The distances of the adjunct SNPs (Kb) are also shown.

and 181 females with mean age of 40.2 years [SD 12.0]). In addition, subjects who were examined in our previous study on the XBP1 gene (Kakiuchi et al 2003), were included in this study; 83 patients with bipolar disorder (27 males and 56 females with mean age of 48.1 years [SD 14.6] and mean age of onset of 35.9 years [SD 15.1], 57 bipolar I [23 males and 34 females] and 26 bipolar II patients [4 males and 22 females]) and 97 healthy controls (51 males and 46 females with mean age of 38.8 years [SD 13.4]). All the subjects were biologically unrelated Japanese. Consensus diagnosis was made for each patient by at least two trained psychiatrists according to the DSM-IV criteria, based on all available information, including clinical interview, medical records and other research assessments. Controls were healthy volunteers who had no current or past contact to psychiatric services. Subjects with significant medical problems, history of head trauma, neurosurgery and alcohol or substance abuse were excluded. After description of the study, written informed consent was obtained from every subject. The study protocol was approved by institutional ethical committees (Showa University School of Medicine, RIKEN Brain Science Institute and National Center of Neurology and Psychiatry, Tokyo, Japan).

SNP Genotyping

Venous blood was drawn from the subjects and genomic DNA was extracted from whole blood according to the standard procedures. Eleven SNPs (SNP1: rs3761418, SNP2: rs2267012, SNP3: rs2267013, SNP4: rs2267015, SNP5: rs2156921, SNP6: rs2213172, SNP7: rs2267020, SNP8: rs1811017, SNP9: rs140504, SNP10: rs131690, SNP11: rs131702; see figure 1) in the BCR gene were genotyped using the TaqMan 5'-exonuclease allelic discrimination assay, described previously (Hashimoto et al 2004; Hashimoto et al 2005). Briefly, primers and probes for detection of the SNPs are: SNP1: forward primer 5'-GGGAGTGAAACAAAATCTTTGATGGTT-3', reverse primer 5'-ATCAGACTCCCTGCTCTTTGC-3', probe 1 5'-VIC-CTGTCTCAGATTTCCAGMGB-3', and probe 2 5'-FAM-CTGTCTCAGATCTCCAG-MGB-3'; SNP2: forward primer 5'-GCATTTTGCAGAATGTCTTCTCTCA-3', reverse primer 5'-ACACTCAGCTAAGAGGGTTCCT-3', probe 1 5'-VIC-CCCTGTGAAGGAGTG-MGB-3', and probe 2 5'-FAM-CCTGTGGAGGAGTG-MGB-3'; SNP3: forward primer 5'-TCTTTGTCAGCGCTGTGGTT-3', reverse primer 5'- CCCACAACAGCAATAAACTAGCAAA-3', probe 1 5'-VIC- CAGTAAGTTCTTTCCCTACCAAG-MGB-3', and probe 2 5'-FAM- TAAGTTCTTTCCCCACCAAG-MGB-3'; SNP4: forward primer 5'-CCACCCTAGGGCATTTTCCT-3', reverse primer 5'-CCAGCTTTCCACTGTTATGAATACAATG-3', probe 1 5'-VIC-CCCCTTTTCTTTTATGGTAG-MGB-3', and probe 2 5'-FAM-CCCCTTTTCTTTTTTGGTAGMGB-3'; SNP5: forward primer 5'-GGAATAGCAGAGTATCTTTTCAACTAGGTT-3', reverse primer 5'-GGACTTCTGGCCCCTTTCAG-3', probe 1 5'-VIC-CCCCTCAATTGCAC-MGB-3', and probe 2 5'-FAM-CCCCTCAGTTGCAC-MGB-3'; SNP6: forward primer 5'-CTAGCAGCTGTGCTCATGGA-3', reverse primer 5'-AGGCCCAGCTCCTATCCT-3', probe 1 5'-VIC-ATCTCACGTCCTCCC-MGB-3', and probe 2 5'-FAM-AATCTCACCTCCTCCCMGB-3'; SNP7: forward primer 5'-CTCGGTGTTGACTTGACCTTACA-3', reverse primer 5'-GGTGGAGCACCTTTTATCTGAGT-3', probe 1 5'-VIC-CTTTCCGAGCCCATG-MGB-3', and probe 2 5'-TTTCCGCGCCCATG-MGB-3'; SNP8: forward primer 5'-GCCACTTCCTGGAAAGAAAGGT-3', reverse primer 5'-TGAGGTCTGGCTGGTGCTA-3', probe 1 5'-VIC-CTGCCAATAGCCC-MGB-3', and probe 2 5'-CTGCCAGTAGCCC-MGB-3'; SNP9: forward primer 5'-AGCTGGACGCTTTGAAGATCA-3', reverse primer 5'-TGGTGTGCACCTTCTCTCTCT-3', probe 1 5'-VIC-CCAGATCAAGAATGACAT-MGB-3', and probe 2 5'-FAM-CCAGATCAAGAGTGACAT-MGB-3'; SNP10: forward primer 5'-CCTGCCTGCCCAGTCC-3', reverse primer 5'-CCCTGGGTTGCAAGGTCTT-3', probe 1 5'-VIC-CAGGCATATTCCTCA-MGB-3', and probe 2 5'-FAM-CAGGCATGTTCCTCA-MGB-3'; SNP11: forward primer 5'-CAGACTGTGTTCGGGTGACA-3', reverse primer 5'-ACCCGGCACTATCCAGACA-3', probe 1 5'-VIC-CAGGAGCTTGTCCTTAAMGB-3', and probe 2 5'-FAM-CAGGAGCTTGTCATTAA-MGB-3'. PCR cycling conditions were: at 95°C for 10 min, 45 cycles of 92°C for 15 sec and 60°C for 1 min.

Statistical Analysis

Statistical analysis of association studies was performed using SNPAlyse software (DYNACOM, Yokohama, Japan). The presence of Hardy-Weinberg equilibrium was examined using the χ^2 test for goodness of fit. Allele distributions between patients and controls were analyzed by the χ^2 test for independence. The measures of linkage disequilibrium (LD), denoted as D' and r^2, were calculated from the haplotype frequency using Expectation-Maximization algorithm. Case-control haplotype analysis was performed by the permutation method to obtain empirical significance (Good 2000). The global p-values represent the overall significance using the χ^2 test when the observed versus expected frequencies of all the haplotypes are considered together. The individual haplotypes were tested for association by

grouping all others together and applying the χ^2 test with 1 *df*. Calculations of *p*-values were based on 10,000 replications. All p-values reported are two tailed. Statistical significance was defined at $p < .05$.

The population homogeneity was assessed using STRUCTURE software (http://pritch.bsd.uchicago.edu/software.html) (Pritchard et al 2000) with eight SNPs, as described previously (Yamada et al 2004). In the application of the Markov chain Monte Carlo method, 1,000,000 replications were used for the burn-in period of the chain and for parameter estimation. Analysis was run at K = 1, 2, 3, 4, and 5. From these results, best estimate of K was found by calculating posterior probabilities, Pr (K = 1, 2, 3, 4, or 5).

Results

The genotype distributions of all the eleven SNPs were in Hardy-Weinberg equilibrium for both the controls and patients with bipolar disorder and major depressive disorder (data not shown). Allele frequencies of the eleven SNPs among the patients and controls are shown in Table 1. The minor alleles of SNP9, SNP10 and SNP11 were in excess in our total bipolar patients when compared to controls (SNP9: $\chi^2 = 7.73$, $df = 1$, $p = .0054$, odds ratio = 1.45 95% CI 1.11–1.84; SNP10: $\chi^2 = 7.48$, $df = 1$, $p = .0063$, odds ratio = 1.50 95% CI 1.14–2.03; SNP11: $\chi^2 = 9.05$, $df = 1$, $p = .0026$, odds ratio = 1.49 95% CI 1.15–1.93), while significant association of the other eight SNPs was not observed with the overall bipolar patients (Table 1). When we examined bipolar I and II separately, there were significant differences in the allele frequency for ten SNPs between patients with bipolar II disorder and controls, while there was a significant difference for one SNP between those with bipolar I disorder and controls (Table 1). Then, we examined a possible association between major depression and the BCR gene. A significant difference in the allele frequency was found for six SNPs between patients with major depressive disorder and controls, and for seven SNPs between total patients with mood disorders and controls (Table 1).

We then focused on the association between the SNPs in the BCR gene and bipolar II disorder. The frequencies of minor allele carriers of the eleven SNPs were compared with major allele homozygotes, as we assumed that minor alleles might have a dominant effect for developing the disease. Nine out of eleven SNPs were significantly associated with bipolar II disorder (Table 2). The smallest *p*-value was obtained in SNP9, N796S missense polymorphism. The S796 allele was significantly more frequent in the bipolar II patients when compared to the controls ($\chi^2 = 8.58$, $df = 1$, $p = .0046$, odds ratio = 3.1, 95% CI 1.53–8.76).

To further analyze the haplotype structure in Japanese population, we computed *D'* and r^2 values for all combinations of the eleven SNPs spanning the BCR locus at an average density of 12.3kb (Table 3). Forty-nine of 55 of possible 2-marker haplotype analysis for all combinations of the eleven SNPs yielded globally significant evidence for association ($p < .05$). That high population of the associated haplotypes is not surprising given the nonindependence of the markers, suggesting that Bonferroni correction might not be appropriate. Adjacent combinations of up to ten markers were examined for association with bipolar II disorder. Six of the nine possible 3-marker haplotype revealed significant evidence for association, as did six of eight of the 4-marker haplotypes and four of seven of 5-marker haplotypes. In total, more than 70% of all possible haplotypes showed the results that gave global significance at $p < .05$. Notably, all the possible combinations of haplotypes including SNP9 (N796S) were associated with bipolar disorder.

Table 1. Allele Distributions for 11 SNPs in the BCR Gene Among Patients With Bipolar Disorder and With Major Depression and Controls

		Controls	BP			BPI			BPII			MDD			Total cases		
SNP-ID	SNP	*n* = 351	*n* = 171	*p* Value	OR	*n* = 102	*p* Value	OR	*n* = 69	*p* Value	OR	*n* = 329	*p* Value	OR	*n* = 500	*p* Value	OR
SNP1	A/G	.349	.395	ns	—	.343	ns	—	.471	.0066	1.66	.415	.012	1.32	.408	.014	1.29
SNP2	A/G	.405	.456	ns	—	.402	ns	—	.536	.0042	1.70	.474	.0097	1.33	.468	.0095	1.29
SNP3	A/G	.261	.295	ns	—	.230	ns	—	.391	.0018	1.82	.310	.044	1.27	.305	.047	1.24
SNP4	T/A	.289	.319	ns	—	.245	ns	—	.428	.0013	1.84	.347	.023	1.30	.337	.037	1.25
SNP5	A/G	.292	.319	ns	—	.245	ns	—	.428	.0017	1.81	.347	.031	1.29	.337	.050	1.23
SNP6	G/C	.298	.330	ns	—	.255	ns	—	.442	.0009	1.87	.353	.031	1.28	.345	.040	1.24
SNP7	G/T	.423	.477	ns	—	.436	ns	—	.536	.0144	1.58	.438	ns	—	.451	ns	—
SNP8	A/G	.051	.061	ns	—	.064	ns	—	.058	ns	—	.061	ns	—	.061	ns	—
SNP9	A/G	.481	.573	.0054	1.45	.534	ns	—	.630	.0014	1.84	.523	ns	—	.540	.018	1.26
SNP10	A/G	.218	.295	.0062	1.50	.270	ns	—	.333	.0036	1.79	.236	ns	—	.256	ns	—
SNP11	T/G	.393	.491	.0026	1.49	.480	.026	1.43	.507	.013	1.59	.386	ns	—	.422	ns	—

Minor allele frequencies in controls are shown. OR, odds ratio; BP, bipolar disorder; MDD, Major depressive disorder; SNP, single nucleotide polymorphism; BCR, breakpoint cluster region.

Table 2. Genotype Distributions for the SNPs in the BCR Gene Among the Patients With Bipolar II and Controls

	Controls			BPII			2/2 and 1/2 vs. 1/1	
SNP-ID	1/1	1/2	2/2	1/1	1/2	2/2	*p* Value	OR (95% CI)
SNP1	151 (43.0%)	155 (44.2%)	45 (12.8%)	19 (27.5%)	35 (50.7%)	15 (21.7%)	.017	1.99 (1.16–3.84)
SNP2	123 (35.0%)	172 (49.0%)	56 (16.0%)	15 (21.7%)	34 (49.3%)	20 (29.0%)	.031	1.94 (1.10–3.78)
SNP3	190 (54.1%)	139 (39.6%)	22 (6.3%)	26 (37.7%)	32 (46.4%)	11 (15.9%)	.012	1.95 (1.19–3.49)
SNP4	171 (48.7%)	157 (44.7%)	23 (6.6%)	22 (31.9%)	35 (50.7%)	12 (17.4%)	.010	2.03 (1.19–3.61)
SNP5	170 (48.4%)	157 (44.8%)	24 (6.8%)	22 (31.9%)	35 (50.7%)	12 (17.4%)	.012	2.01 (1.13–3.65)
SNP6	165 (47.0%)	163 (46.4%)	23 (6.6%)	21 (30.4%)	35 (50.7%)	13 (18.8%)	.011	2.03 (1.19–3.61)
SNP7	115 (32.8%)	175 (49.9%)	61 (17.4%)	15 (21.7%)	34 (49.3%)	20 (29.0%)	.070	1.75 (1.01–3.62)
SNP8	315 (89.7%)	36 (10.3%)	0 (0%)	61 (88.4%)	8 (11.6%)	0 (0%)	ns	—
SNP9	91 (25.9%)	182 (51.9%)	78 (22.2%)	7 (10.1%)	37 (53.6%)	25 (36.2%)	.0046	3.10 (1.53–8.76)
SNP10	215 (61.3%)	119 (33.9%)	17 (4.8%)	32 (46.4%)	28 (40.6%)	9 (13.0%)	.022	1.83 (1.08–3.19)
SNP11	128 (36.5%)	170 (48.4%)	53 (15.1%)	15 (21.7%)	38 (55.1%)	16 (23.2%)	.018	2.07 (1.20–4.02)

Allele 1 represents a major allele in each SNP. SNP, single nucleotide polymorphism; BCR, breakpoint cluster region; BP, bipolar disorder; OR, odds ratio.

Adjacent marker combinations yielding global evidence for association at $p < .005$ are presented in figure 2. The haplotypes that yielded global evidence for significant association at this level included the SNP9 and SNP10. The haplotypes that yielded the strongest global evidence for significant association consisted of markers SNP8-SNP9-SNP10-SNP11 (global permutation p value = .00041, 100,000 simulations). Given this result, we tested the contribution of individual haplotypes to the global result. The lowest p-value was obtained for the difference in the frequency of 1-2-2-2 haplotype (SNP8-SNP9-SNP10-SNP11), which was enriched in patients with bipolar II disorder compared with controls (estimated frequencies: patients 30.2% vs controls 16.7%, permutation p value = .0002). Another individual haplotype that yielded the evidence for significant association was 1-1-1-1 haplotype, which occurs at a frequency of 33.5% in the patients and 45.7% in the controls (permutation p value = .0066).

Discussion

We found a significant association between genetic variations of the BCR gene and bipolar disorder and major depressive disorder in a Japanese population. Our data suggest that the BCR gene is associated with bipolar II rather than bipolar I disorder. The weaker association with major depression compared to bipolar II disorder might be due to some patients with major depression who could develop bipolar II disorder in the future and/or the susceptibility both for bipolar II and major depression. The diagnostic category of bipolar II disorder is defined as less severe manic symptoms compared with bipolar I disorder. Bipolar II patients tend to have more previous episodes, including both depressive and hypo-manic, but less hospitalization and psychotic symptoms (Vieta et al 1997). Some studies showed that bipolar disorder is likely to be a quantitative trait (bipolar I – bipolar II – major depression), while other studies argued that bipolar II may be genetically distinct to bipolar I disorder (Kelsoe 2003). Bipolar I and bipolar II were of approximately similar prevalence in the first degree relatives of bipolar I probands (8.5 vs. 6.1%, respectively), while bipolar II was significantly more prevalent among the first degree relatives of bipolar II probands (3% vs. 30%, respectively) (Coryell et al 1984). These data suggest that some genes confer susceptibility to both bipolar I and bipolar II, while a separate and more common set of genes predisposes preferentially or exclusively to bipolar II disorder. Kelsoe proposed a model of bipolar genetics that some of the genes involved are specific for each of the phenotypes considered bipolar I, bipolar II and major depression and others are less specific and may predispose individuals to either bipolar disorders or major depression (Kelsoe 2003).

The XBP1 gene, which has been reported to be associated with bipolar disorder, is also located on the chromosome 22q (Kakiuchi et al 2003). However, the physical distance between the XBP1 and BCR gene is approximately 5.5 Mb, and the D' value between the -116C/G polymorphism of the XBP1 gene and

Table 3. Marker-to-Marker LD for All the Combinations of the 11 SNPs in the BCR Gene

—	SNP1	SNP2	SNP3	SNP4	SNP5	SNP6	SNP7	SNP8	SNP9	SNP10	SNP11
SNP1	—	.766[a]	.610[a]	.483[a]	.493[a]	.480[a]	.311[a]	.000	.240[b]	.002[b]	.067[a]
SNP2	.985[a]	—	.475[a]	.576[a]	.585[a]	.557[a]	.360[a]	.001	.279[a]	.009[b]	.081[a]
SNP3	.963[a]	.957[a]	—	.785[a]	.801[a]	.765[a]	.148[a]	.001	.114[b]	.026[a]	.015[a]
SNP4	.797[a]	.981[a]	.952[a]	—	.986[a]	.946[a]	.192[a]	.001[b]	.154[b]	.041[a]	.018[b]
SNP5	.800[a]	.981[a]	.968[a]	1.000[a]	—	.959[b]	.191[a]	.001[b]	.152[b]	.039[a]	.018[a]
SNP6	.779[a]	.945[a]	.959[a]	.993[a]	.993[b]	—	.193[a]	.001[a]	.153[b]	.040[a]	.016[b]
SNP7	.653[a]	.624[a]	.554[a]	.588[a]	.582[a]	.578[a]	—	.040[a]	.693[a]	.113[a]	.360
SNP8	.095	.137	.089	.086[b]	.078[b]	.106[a]	1.000[a]	—	.058[a]	.000	.006
SNP9	.644[b]	.617[a]	.549[b]	.593[b]	.585[b]	.579[b]	.937[a]	1.000[a]	—	.118[a]	.344[b]
SNP10	.064[b]	.147[b]	.183[a]	.244[a]	.241[a]	.247[a]	.546[a]	.105	.627[a]	—	.430[a]
SNP11	.285[a]	.291[a]	.165[a]	.169[b]	.170[a]	.154[b]	.638	.424	.702[b]	1.000[a]	—

For each pair of markers, the standardized D' is shown below the diagonal, and r^2 above the diagonal.
LD, linkage disequilibrium; SNP, single nucleotide polymorphism; BCR, breakpoint cluster region.
[a] $p < .05$.
[b] $p < .01$.

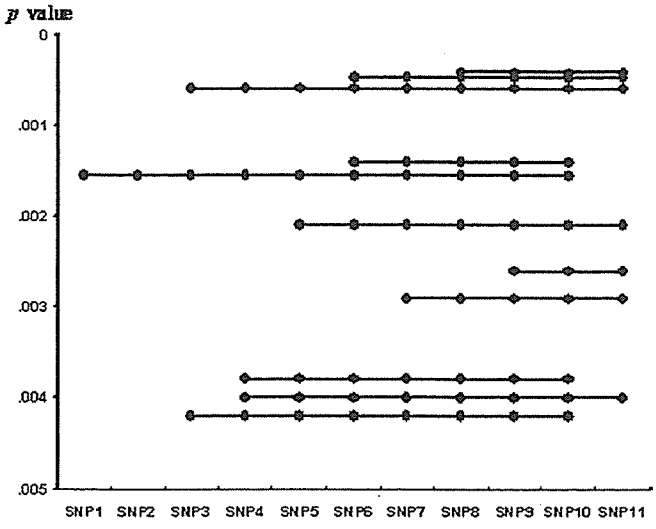

Figure 2. Plot of haplotypes showing global association to bipolar II disorder ($p < .005$). The x-axis scale is nonlinear in order to allow easy visualization of the different haplotypes. SNP, single nucleotide polymorphism.

any SNPs in the BCR gene is less than .3, and r^2 values are less than .004 both in patients and controls groups, suggesting that genetic association between bipolar disorder and both genes might be independent. In addition, we did not observe the evidence for the association between the -116C/G SNP and bipolar disorder in the population of the present study, although approximately half of the subjects in this study were overlapped with the Kakiuchi's study (G allele frequencies, Kakiuchi's, case: .71, control: .64; present study, case: .68, control: .70) (Kakiuchi et al 2003).

Our data suggest the existence in our sample of a risk haplotype and a protective haplotype consisting of SNP8-SNP9-SNP10-SNP11. We intended to determine the haplotype block structure of the region (Gabriel et al 2002), examining eleven SNPs at an average density of 12.3kb across the BCR gene. Unfortunately, we were not able to define the obvious haplotype blocks in a Japanese sample, because of the complex results of marker to marker LD. It is of interest how genetic variation might affect BCR function/expression. Although we have no evidence whether any of the SNPs in our haplotypes are functional, SNP9 associated with bipolar disorder might be functional as this SNP gives rise to an amino acid substitution of N796S in the functional domain, pleckstrin homology (PH) domain, of the BCR protein. This SNP might account for the susceptibility for bipolar disorder, as the individuals carrying the S796 allele were most significantly in excess in bipolar II patients in our study ($p = .0046$, odds ratio = 3.1). Alternatively, an unknown functional polymorphism, which is in LD with the SNPs and/or haplotypes, may be responsible for biologic susceptibility for bipolar II. Further work, e.g. dense mapping in the BCR gene, and functional analysis of N796S missense polymorphism, will be required to resolve this issue.

The function of the normal BCR gene product remains unclear, although the BCR-ABL fusion protein, which causes certain human leukemias, has been extensively studied (Pane et al 2002). The BCR gene encodes a 1271 amino acid protein containing several functional domains: a serine/threonine protein kinase domain, a Dbl homology domain, a PH domain and a Rho GTPase-activating protein domain. This protein acts as a serine/threonine kinase, a GTPase-activating protein for p21rac, and a Rho GTPase guanine nucleotide exchange factor (Diekmann et al 1991; Korus et al 2002; Maru and Witte 1991). The PH domain is a 100–120 amino acid protein module best known for its ability to bind phosphatidylinositol (Lemmon et al 2002). PH domain-containing proteins specifically recognize 3-phosphorylated phosphatidylinositol, allowing them to drive membrane recruitment in response to phosphatidylinositol 3-kinase activation. A dysfunction in the phosphatidylinositol signal transduction pathway appears to be implicated in the pathophysiology of bipolar disorder, e.g. increased intracellular calcium responsiveness and protein kinase C activity in platelets and transformed lymphoblasts, and decreased inositol levels in frontal cortex of the postmortem brain (Shimon et al 1997; Soares and Mallinger 1997). The N796S missense polymorphism in the PH domain of BCR could affect its binding activity to phosphatidylinositol, and then alter the phosphatidylinositol signal transduction pathway. Both asparagine and serine residue are neutral amino acids (polar amino acids), while only serine residue could be phosphorylated. Additionally, the sequence around N796 is evolutionarily conserved across several species, including fruit fly, African clawed frog, mouse, rat, and human. As S796 is unique for humans, this polymorphism might be associated with the higher brain function in humans.

As a common action of mood stabilizers is to inhibit the collapse of neuronal growth cone via depletion of inositol (Williams et al 2002), neuronal growth cone formation is likely to be involved in the pathogenesis of bipolar disorder. BCR is a RhoGAP protein, which inactivates the Rho GTPase. Rho GTPase proteins activate their effecters, which control cytoskeletal organization (Kaibuchi et al 1999), leading to the motile behavior of the neurite and growth cone (Huber et al 2003). As Rho associated kinase, one of the Rho effectors, regulates the dynamic reorganization of cytoskeletal proteins, such as actin, neurofilament and glial fibrillary acidic protein (Amano et al 1997; Hashimoto et al 1998; Kosako et al 1997), it is worth investigating the possible effect of BCR, including N796S amino acid change, on the growth cone formation and Rho associated kinase.

We have firstly demonstrated the possible association between the BCR gene and bipolar disorder in a Japanese population. The limitation of this work is that there were differences with regard to mean age and gender distribution between patients and controls. Thus, we assessed the population homogeneity and did not detect any evidence for such stratification in our samples with a Pr ($K = 1$) > .99. However, a false-positive association due to the small sample size of the bipolar II disorder patients could not be excluded in our study. Further investigations are warranted to confirm our findings in other samples.

We thank Tomoko Shizuno and Reiko Fujita for technical assistance and Dr. Kazuo Yamada and Kozo Kaibuchi for helpful discussions.

This work was supported by Grants-in-Aid from the Japanese Ministry of Health, Labor and Welfare, the Japanese Ministry of Education, Culture, Sports, Science and Technology, the Uehara Memorial Foundation, and Japan Foundation for Neuroscience and Mental Health.

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doi:10.1093/brain/awh702 *Brain* (2006), **129**, 399–410

The association between the Val158Met polymorphism of the catechol-*O*-methyl transferase gene and morphological abnormalities of the brain in chronic schizophrenia

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The catechol-O-methyl transferase (COMT) gene is considered to be a promising schizophrenia susceptibility gene. A common functional polymorphism (Val158Met) in the COMT gene affects dopamine regulation in the prefrontal cortex (PFC). Recent studies suggest that this polymorphism contributes to poor prefrontal functions, particularly working memory, in both normal individuals and patients with schizophrenia. However, possible morphological changes underlying such functional impairments remain to be clarified. The aim of this study was to examine whether the Val158Met polymorphism of the COMT gene has an impact on brain morphology in normal individuals and patients with schizophrenia. The Val158Met COMT genotype was obtained for 76 healthy controls and 47 schizophrenics. The diagnostic effects, the effects of COMT genotype and the genotype-diagnosis interaction on brain morphology were evaluated by using a voxel-by-voxel statistical analysis for high-resolution MRI, a tensor-based morphometry. Patients with schizophrenia demonstrated a significant reduction of volumes in the limbic and paralimbic systems, neocortical areas and the subcortical regions. Individuals homozygous for the Val-COMT allele demonstrated significant reduction of volumes in the left anterior cingulate cortex (ACC) and the right middle temporal gyrus (MTG) compared to Met-COMT carriers. Significant genotype-diagnosis interaction effects on brain morphology were noted in the left ACC, the left parahippocampal gyrus and the left amygdala-uncus. No significant genotype effects or genotype-diagnosis interaction effects on morphology in the dorsolateral PFC (DLPFC) were found. In the control group, no significant genotype effects on brain morphology were found. Schizophrenics homozygous for the Val-COMT showed a significant reduction of volumes in the bilateral ACC, left amygdala-uncus, right MTG and left thalamus compared to Met-COMT schizophrenics. Our findings suggest that the Val158Met polymorphism of the COMT gene might contribute to morphological abnormalities in schizophrenia.

Keywords: schizophrenia; polymorphism; COMT; ACC; DLPFC

Abbreviations: ACC = anterior cingulate cortex; COMT = catechol-O-methyl transferase; DLPFC = dorsolateral prefrontal cortex; FDR = false discovery rate; IQ = intelligence quotient; JART = Japanese version of National Adult Reading Test; ROI = region of interest; SPM = statistical parametric mapping; TBM = tensor-based morphometry

Received July 15, 2005. Revised September 21, 2005. Accepted October 27, 2005. Advance Access publication December 5, 2005

Introduction

Schizophrenia is a severe neuropsychiatric disorder with deficits of multiple domains of cognitive functions, volition and emotion. Family and twin studies have provided cumulative evidence for a genetic basis of schizophrenia (Kendler, 1983; McGue *et al.*, 1983; Sullivan *et al.*, 2003); however, identification of the underlying susceptibility loci has been limited. Collective data have suggested that the aetiology of schizophrenia involves the interplay of complex polygenic influences and environmental risk factors operating on brain maturational processes (Harrison *et al.*, 2005).

In vivo neuroimaging studies have demonstrated that brain abnormalities should play an important role in the pathophysiology of schizophrenia. Structural MRI studies have demonstrated relatively consistent brain abnormalities in patients with schizophrenia, such as enlargement of the ventricular system and regional volume decrease in the temporal lobe structures (Gaser *et al.*, 2001; Okubo *et al.*, 2001; Shenton *et al.*, 2001; Davidson and Heinrichs, 2003). Studies with schizophrenics and their healthy siblings demonstrate that even healthy siblings share some of morphological abnormalities observed in schizophrenia (Steel *et al.*, 2002; Gogtay *et al.*, 2003). A recent morphological MR study revealed that a common polymorphism of the brain-derived neurotrophic factor, one of the well-known schizophrenia susceptibility genes, affected the anatomy of the hippocampus and prefrontal cortex (PFC) in healthy individuals (Pezawas *et al.*, 2004). Furthermore, some studies have suggested that environmental factors interact with genetic factors (Cannon *et al.*, 1993; Nelson *et al.*, 2004). For example, obstetric complications are well known non-genetic risk factors of schizophrenia. However, a previous study suggested that obstetric complications might induce brain morphological abnormalities in schizophrenics and their siblings, but not in comparison with subjects at low genetic risk for schizophrenia (Cannon *et al.*, 1993). These facts suggest that genetic factors should have considerable impact on brain morphology in patients with schizophrenia.

Catechol-O-methyl transferase (COMT) is a promising schizophrenia susceptibility gene because of its role in monoamine metabolism (Goldberg *et al.*, 2003; Stefanis *et al.*, 2004; Harrison *et al.*, 2005). A common single nucleotide polymorphism (SNP) of the COMT gene producing an amino acid substitution of methionine (met) to valine (val) at position 108/158 (Val158Met) affects dopamine regulation in the PFC (Palmatier *et al.*, 1999). This polymorphism impacts on the stability of the enzyme, such that the Val-COMT allele has significantly lower enzyme activity than the Met-COMT allele (Weinberger *et al.*, 2001; Chen *et al.*, 2004). Several studies have revealed that the Val-COMT allele is associated with poorer performances, compared to the Met-COMT allele, in cognitive tasks of frontal function such as the Wisconsin Card Sorting Test (WCST) and N-back task (Egan *et al.*, 2001; Weinberger *et al.*, 2001; Goldberg *et al.*, 2003). The underlying mechanism of such behavioural differences may be related to lower prefrontal dopamine levels arising from higher dopamine catabolism mediated by the Val-COMT allele (Chen *et al.*, 2004; Tunbridge *et al.*, 2004).

The results of studies on the association between the Val158Met polymorphism and schizophrenia have, however, been controversial (Daniels *et al.*, 1996; Kunugi *et al.*, 1997; Ohmori *et al.*, 1998; Norton *et al.*, 2002; Galderisi *et al.*, 2005; Ho *et al.*, 2005). The result of a meta-analysis was even more inconclusive (Fan *et al.*, 2005). Such inconsistency was also found in associations between frontal functions and the Val158Met polymorphism (Egan *et al.*, 2001; Weinberger *et al.*, 2001; Goldberg *et al.*, 2003; Ho *et al.*, 2005). The possible morphological changes due to the COMT gene might be present and play a role in susceptibility to schizophrenia and in giving rise to impaired frontal functions. However, morphological changes underlying functional impairments remain to be clarified.

A recent advancement of methods for MR volumetry, such as voxel-based morphometry and deformation-based morphometry [or tensor-based morphometry (TBM)], allows us to explore and analyse brain structures of schizophrenics (Wright *et al.*, 1995; Gaser *et al.*, 2001). Using TBM techniques, we investigated the association between the Val158-Met polymorphism of the COMT gene and brain morphology in normal individuals and patients with schizophrenia. The aim of this study was to clarify whether there are significant genotype and/or genotype-disease interaction effects on brain morphology.

Methods

Subjects

Seventy-six healthy subjects and forty-seven patients with schizophrenia participated in the study. All the subjects were biologically unrelated Japanese. Written informed consent was obtained from all the subjects in accordance with ethical guidelines set by a local ethical committee. All normal subjects were screened using a questionnaire on medical history and excluded if they had neurological, psychiatric or medical conditions that could potentially affect the CNS, such as substance abuse or dependence, atypical headache, head trauma with loss of consciousness, asymptomatic or symptomatic cerebral infarctions detected by T_2-weighted MRI, hypertension, chronic lung

disease, kidney disease, chronic hepatic disease, cancer, or diabetes mellitus. The patients were diagnosed on the basis of DSM-IV criteria, information from medical records and a clinical interview. All patients were stable and/or partially remitted at the time of MR measurement and neuropsychological tests.

According to genotypes, each group (control and schizophrenia) was categorized into three groups; the homozygous Val-COMT group (control: n = 38, two were left-handed, schizophrenia: n = 19, one was left-handed), the Val/Met-COMT group (control: n = 25, three were left-handed, schizophrenia: n = 22, all were right-handed) and the remaining homozygous Met-COMT group (control: n = 13, all were right-handed, schizophrenia: n = 6, all were right-handed). Because of the small number of subjects with homozygous Met-COMT, the Val/Met-COMT and homozygous Met-COMT groups were combined and treated as one group, the Met-COMT carriers. Table 1 shows the characteristics of each group. All groups were of comparable age, gender (χ^2 test, df = 3, P = 0.38) and handedness (χ^2-test, df = 3, P = 0.53). No genotype effects and genotype-diagnosis interaction effects were found in years of education, scores of full scale Intelligence Quotient (IQ) and scores of premorbid IQ [Japanese version of National Adult Reading Test (JART) score], however, patients who had fewer years of education ($P < 0.0001$), had lower scores of both full scale IQ and JART ($P < 0.001$). The duration of illness, medication and hospitalization, the age at disease onset and drug dose (chlorpromazine equivalent) of those homozygous for the Val-COMT did not differ from the Met-COMT carriers.

SNP genotyping

Venous blood was drawn from subjects and genomic DNA was extracted from whole blood according to the standard procedures. The Val158Met polymorphism of the COMT gene (dbSNP accession: rs4680) was genotyped using the TaqMan 5′-exonuclease allelic discrimination assay, described previously (Hashimoto *et al.*, 2004, 2005). Briefly, primers and probes for detection of the SNP are: forward primer 5′-GACTGTGCCGCCATCAC-3′, reverse primer 5′-CAGGCATGCACACCTTGTC-3′, probe 1 5′-VIC-TTTCGCTG-GCGTGAAG-MGB-3′ and probe 2 5′-FAM-CGCTGGCATGAAG-MGB-3′. PCR cycling conditions were: at 95°C for 10 min, 50 cycles of 92°C for 15 s and 60°C for 1 min.

MRI procedures

All MR studies were performed on a 1.5 tesla Siemens Magnetom Vision plus system. A three dimensional (3D) volumetric acquisition of a T_1-weighted gradient echo sequence produced a gapless series of thin sagittal sections using an MPRage sequence (TE/TR, 4.4/11.4 ms; flip angle, 15°; acquisition matrix, 256 × 256; 1 NEX, field of view, 31.5 cm; slice thickness, 1.23 mm).

Image analysis (TBM)

The basic principle of TBM is to analyse the local deformations of an image and to infer local differences in brain structure. In TBM, MRI scans of individual subjects are mapped to a template image with three-dimensional (3D) non-linear normalization routines. Local deformations were estimated by a univariate Jacobian approach. The basic principle of TBM is the same as a method used in a previous report described as deformation-based morphometry (Gaser *et al.*, 2001). Firstly, inhomogeneities in MR images were corrected using a bias correction function in statistical parametric mapping (SPM2), then the corrected image was scalp-edited by masking with a probability image of brain tissue obtained from each image using a segmentation function in SPM2. Using a linear normalization algorithm in SPM2, all brains were resized to a voxel size of 1.5 mm and adjusted for orientation and overall width, length and height (Fig. 1A). Therefore, brains were transformed to the anatomical space of a template brain whose space is based on Talairach space (Talairach and Tournoux, 1988). Subsequent non-linear normalization introduced local deformations to each brain to match it to the same scalp-edited template brain (Fig. 1C). The non-linear transformation was done using the high-dimension-warping algorithm (Ashburner and Friston, 2004). After the high dimensional warping, each image (Fig. 1B) looks similar to the template (Fig. 1C). Figure 2 demonstrated a mean MR image of 76 controls (left) and a mean MR image of 47 schizophrenics after high dimensional warping (Fig. 2). We obtained 3D deformation fields for every brain (Fig. 1D). Each of these 3D deformation fields consists of displacement vectors for every voxel, which describe the 3D displacement needed to locally deform the brain to match it to the template. We calculated the Jacobian determinants to obtain voxel by voxel parametric maps of local volume change relative to the template brain (Fig. 1E). The local Jacobian determinant is a parameter commonly used in continuum mechanics (Gurtin, 1987), which characterizes volume changes, such as local shrinkage or enlargement caused by warping. The parametric maps of Jacobian determinants were analysed using SPM2, which implements a 'general linear model'. To test hypotheses about regional population effects and interaction, data were analysed by an analysis of covariance (ANCOVA) without global normalization. There was no significant difference in age among the four groups, however, patients with schizophrenia, particularly those homozygous for the Val-COMT allele, were older than controls. Therefore, we treated age and years of education and scores of JART as nuisance variables. Since TBM explores the entire brain (grey matter, CSF space and white matter) at once, the search volume of TBM has a large number of voxels and since our interest was in morphological changes in the grey matter and CSF space, we excluded white matter tissue from analyses by using an explicit mask (Fig. 1F). We used $P < 0.001$, corrected for multiple comparisons with false discovery rate (FDR) <0.05 as a statistical threshold. The resulting sets of t values constituted the statistical parametric maps {SPM (t)}. Firstly, we estimated the main effects, the genotype effect in total subjects (the Val/Val-COMT versus the Met-COMT carriers) and the diagnostic effect (schizophrenia versus controls) and then the genotype-diagnosis interaction effect was estimated. Furthermore, the effects of genotypes in each group (controls carrying the Val/Val-COMT gene versus controls carrying the Met-COMT gene and schizophrenics carrying the Val/Val-COMT gene versus schizophrenics carrying the Met-COMT gene) were estimated within the ANCOVA design matrix. Anatomical localization accorded both to MNI coordinates and Talairach coordinates obtained from M. Brett's transformations (www.mrc-cbu.cam.ac.uk/Imaging/mnispace.html) and are presented as Talairach coordinates (Talairach and Tournoux, 1988). Since previous studies have demonstrated the association between the Val158Met polymorphism and the dorsolateral PFC (DLPFC), we applied an additional hypothesis-driven region of interest (ROI) method to test regional population effects in the DLPFC. For this ROI analysis, we used the Wake Forest University PickAtlas (Maldjian *et al.*, 2003) within the ANCOVA design matrix for SPM analysis. We set $P < 0.05$ (uncorrected) with a small volume correction ($P < 0.05$ within the ROI) to assess grey matter volume changes in the DLPFC (Brodmann area 46, 9 and 8).