Table 1 *HSPA5* haplotype frequencies in bipolar disorder

	Japanese case-control samples					NIMH trios (n = 88)		
		All cases (n = 195)		With family history (n = 67)				
Haplotype	Controls (n = 254)	Cases	P	Cases	P	Freq. T	Freq. NT	P
1: C-del-C	0.116	0.135	0.375	0.141	0.426	0.074	0.069	0.835
2: C-del-T	0.076	0.128	0.010	0.201	0.000084	0.0057	0	0.239
3: C-G-C	0.368	0.325	0.185	0.276	0.043	0.38	0.41	0.583
4: T-del-T	0.439	0.410	0.388	0.380	0.222	0.52	0.51	0.914

Other haplotypes estimated from the NIMH samples were less than 2%. Haplotypes consist of three polymorphisms (–307C→T-rs3216733-rs12009). Freq. T or Freq. NT indicates the frequency of the transmitted or nontransmitted haplotype, respectively. P values were calculated by COCAPHASE.

transcription start site), rs3216733 (Gdel), and rs12009 (C→A)) in Japanese case-control samples (described previously[1]), we found that haplotype 2 (C-del-T) was significantly associated with bipolar disorder (P = 0.010). This association was stronger in affected individuals with family history (P = 0.000084; Table 1). This risk haplotype was extremely rare in the NIMH trio samples, and no association was found (Table 1).

These findings suggest that genetically determined interindividual variability of ER stress–response does relate to bipolar disorder, that there may be functional polymorphisms in other ER stress–response–related genes, in addition to the –116 polymorphism of *XBP1*, and that genetic risk factors may differ among populations.

The antimalaria drug mefloquine, which often causes an episode of depression or mania in susceptible individuals, was recently reported to cause ER stress in the brain[2]. Further investigations of the ER stress–response signaling system in the pathophysiology of bipolar disorder is warranted.

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A national DNA bank in The Gambia, West Africa, and genomic research in developing countries

To the editor:

The Gambian National DNA Bank, the first National Bio-Bank developed in Africa, was funded in November 2000 by the Medical Research Council (MRC) as one of 14 DNA collection sites established to study the genetics of complex diseases. One of these sites is housed at the MRC Laboratories in The Gambia and has a special, though not exclusive, focus on malaria, HIV and tuberculosis. Additional projects include analyses of genome diversity in West African populations and a collection of twin-sister pairs to study the genetic basis of dizygotic twins (~2% of live births in the country). So far, more than 30,000 DNA samples have been collected, with many ongoing studies and more planned.

For the first time in a sub-Saharan country, a centralized structure and database for archiving DNA samples has been created, in collaboration with the Jean Dausset Foundation-CEPH. The bank is regulated by guidelines (**Supplementary Note** online) for sample collection, archiving, data storage and privacy protection, which were developed and approved by the MRC, the MRC Laboratories Scientific Coordinating Committee and by the Gambia Government/MRC Joint Ethics Committee. The Guidelines, which are enforced by these Committees, stemmed from the need to adapt to the local reality the many existing recommendations on bio-banking, privacy protection, genetics research and, generally, on medical research in developing countries (http://www3.who.int/whosis/genomics/pdf/genomics08.pdf, http://www.mrc.ac.uk/pdf-devsoc.pdf, http://www.mrc.ac.uk/pdf-tissue_guide_fin.pdf, http://www.nuffieldbioethics.org/publications/pp_0000000013.asp).

The Gambian DNA Bank promotes sharing of information and resources with centers around the world, and one of its ultimate goals is health improvement. In the short term, benefits should accrue to the participants in the studies. A recent example is a large project on genetic and environmental factors for susceptibility to tuberculosis, designed as a household association and family-based study and carried out in The Gambia, Guinea-Bissau and Guinea-Conakry[1]. The project focused on the systematic detection of tuberculosis cases in the families of individuals with tuberculosis and controls. Clinical services

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Short Communication

Association of the *XBP1* –116C/G polymorphism with schizophrenia in the Japanese population

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Abstract Schizophrenia and bipolar disorder share some clinical features and linkage studies have shown that several loci are common. Recently, the authors found that the –116C→G substitution in the promotor region of *XBP1*, a pivotal gene in endoplasmic reticulum (ER) stress response, causes the impairment of ER stress response, and that the –116C/C genotype is a protective factor; in other words the presence of the G allele increases the risk for bipolar disorder. The gene is located on 22q12.1, which is also linked with schizophrenia. The polymorphisms were investigated in 234 schizophrenic patients as compared with controls. Significant difference of genotype distribution was observed, which suggested that the –116C/C genotype is a protective factor for both of the major mental disorders.

Key words association study, rheumatoid arthritis, schizophrenia, XBP1.

INTRODUCTION

Schizophrenia is a severe mental illness affecting 1% of the population. Although genetic factors have been shown in family, twin and adoption studies, susceptibility genes or molecular mechanisms of the illness are controversial.[1] Schizophrenia and bipolar disorder are known to share a common genetic background evidenced by linkage and family studies. A case of monozygotic twins, of which one had schizophrenia and the other had bipolar disorder, has been reported.[2] Several loci (18p11, 13q32, 10p14, 22q11–13) are reported to be linked with both of these disorders.[3] We have recently identified that the XBP1 gene is associated with a genetic risk of bipolar disorder.[4]

It is known that XBP1 is a pivotal gene in endoplasmic reticulum (ER) stress response.[5] When unfolded proteins are accumulated in the ER, ER chaperons such as HSPA5 (also called GRP78 or BiP) assist in the re-folding of them. When HSPA5 proteins are consumed and dissociated from ATF6, ATF6 protein is cleaved. Cleaved ATF6 protein induces the expression of target genes harboring ER stress-response element (ERSE), such as *XBP1* and *HSPA5*. In parallel with ATF6 protein cleavage, IRE1 proteins on the ER membrane are dimerized by dissociation of HSPA5 and subsequently splice *XBP1* mRNA. The spliced mRNA encodes active-form XBP1 that strongly induces the expression of chaperons including *HSPA5*, as well as *XBP1* itself. This sequential response is referred to as ER stress response.[5–7] We found that the –116C→G substitution in the promotor region of the *XBP1* gene, losing the binding motif of XBP1 itself, impaired the XBP1 loop in the ER stress response, and that the –116C/C genotype was a protective factor; in other words, having the –116G allele was a risk factor for bipolar disorder.[4]

The *XBP1* gene is located on 22q12.1, one of the common susceptibility loci for schizophrenia and bipolar disorder.[8] In this report we examined the association of this polymorphism with schizophrenia to examine whether this polymorphism is a common

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Table 1. *XBP1* −116C/G polymorphism

	Genotype			Allele	
	C/C *n* (%)	C/G *n* (%)	G/G *n* (%)	C *n* (%)	G *n* (%)
Schizophrenia	17 (0.08)	111 (0.47)	106 (0.45)	145 (0.31)	323 (0.69)
Control	65 (0.14)	195 (0.43)	191 (0.42)	325 (0.36)	577 (0.64)

Genotype frequencies ($P = 0.019$) but not allele frequencies ($P = 0.062$), differ significantly between patients and controls.

protective or risk factor for these two major mental disorders.

METHODS

We genotyped the *XBP1* −116C/G polymorphism in Japanese case–control samples. The patients consisted of 234 unrelated patients followed up at the hospitals or clinics participating in the present study and diagnosed by the authors after repeated clinical interviews according to the *Diagnostic and Statistical Manual of Mental Disorders* (4th edn; DSM-IV) criteria. Written informed consent was obtained from all the subjects. The Ethics Committees of the Brain Science Institute (RIKEN) and participating institutes approved the study.

The *XBP1* −116C/G polymorphism was genotyped by polymerase chain reaction (PCR) amplification with the primers 5′-CGACAGAAGCAGAACTTTAG and 5′-CTGAGGTAATTCTCTGTTAG in a 12.5 mL volume containing LA Taq, deoxynucleotide triphosphates (dNTP) and 2 × GC buffer I (Takara, Shiga, Japan). Amplification conditions consisted of an initial 2 min at 94°C, 35 cycles of 30 s at 94°C, 30 s at 54°C and 1 min at 72°C, followed by a final extension of 2 min at 72°C. Sequencing was performed using a commercial kit (BigDye terminator Cycle Sequencing Ready Reaction Kit, Applied Biosystems, Foster City, CA, USA). The genotype and allele frequencies in patients with schizophrenia were compared with that in 451 unrelated healthy control subjects reported elsewhere.[3] Differences in genotype and allele frequencies were examined by Fisher's exact test.

RESULTS

Genotype distribution was within the Hardy–Weinberg Equilibrium in both groups. There was a significant difference of −116C/G genotype distribution between patients and controls. A similar tendency was also found for allele frequency (Table 1). The C/C genotype was significantly less common (8%) in schizophrenia patients compared with controls (14%). The odds ratio for having the G allele was 2.15 (95%CI: 1.23–3.76). These results suggest that the −116C/C genotype, the protective genotype for bipolar disorder, is also a protective factor for schizophrenia, although the significance is modest and the odds ratio is lower than that for bipolar disorder (4.6; 95%CI: 2.1–10.2).[4]

DISCUSSION

In the present report we have suggested that the −116C/C genotype, a protective factor for bipolar disorder, is also protective for schizophrenia. The molecular function of XBP1 in the brain is not known, but the XBP1 gene is expressed in the brain at relatively high levels.[4,9] The ER stress response cascade or other unidentified downstream genes of XBP1 may be associated with the pathophysiology of both of the mental disorders. Further studies of this cascade have a potential to clarify the molecular mechanism of both disorders.

In contrast, *XBP1* is not only pivotal in ER stress response, but also plays a critical role in plasma cell differentiation of B cells and is highly expressed in the plasma cells of inflammatory synovium of rheumatoid arthritis patients.[10,11] Negative association between schizophrenia and rheumatoid arthritis is a classical and well-replicated finding and a meta-analysis suggested that the prevalence of rheumatoid arthritis in patients with schizophrenia is only 10–30% of the general population.[12] However, the mechanism is unknown.[13] Although the association of schizophrenia with the XBP1 −116C/G polymorphism itself was modest, other polymorphisms in this cascade, in addition to the reduced promotor activity due to −116C/G, may explain this classic but unexplained finding.

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Lack of association between XBP1 genotype and calcium signaling in the platelets of healthy subjects

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Abstract

Dysregulations of calcium (Ca) homeostasis may be involved in the pathophysiology of bipolar disorder. Enhanced Ca response to various agonists in peripheral blood cells is one of a few confirmed biological markers for bipolar disorder. Recently, a polymorphism of XBP1, a pivotal gene in the endoplasmic reticulum (ER) stress response, was shown to contribute to the genetic risk factor for bipolar disorder. Thus, in this study, we examined the relationship between the XBP1 gene polymorphism and the Ca signaling in the platelets of healthy controls. The present results suggest no significant difference in the basal Ca level or 5-HT-induced Ca mobilization among normal subjects with −116C/C, C/G, and G/G genotypes. Further investigations are necessary to examine the relationship in the different peripheral blood cells and/or in larger samples from patients with bipolar disorder.

Keywords: Calcium; XBP1; Endoplamic reticulum; Bipolar disorder; Polymorphism; Healthy subject

Altered calcium (Ca) signaling has been reported in the peripheral blood cells of patients with bipolar disorder. We have reported that serotonin (5-HT)- or thrombin-induced intracellular Ca mobilization is enhanced in the platelets of unmedicated patients with bipolar disorder [10,11,16]. Other researchers also indicated similar findings in platelets [1,3,14,20], in neutrophils [19], and in transformed lymphoblastoid cells [8]. Our longitudinal follow-up study suggested that the enhanced Ca response is trait dependent [11] and that patients with marked increased Ca response are good responders to mood stabilizers such as lithium and valproate [12]. These findings in non-neuronal cells, which could provide clues to the molecular basis of the disease, suggest that the altered Ca mobilization might be involved in the pathophysiology of bipolar disorder. The enhanced Ca response could be due to altered functioning of endoplasmic reticulum (ER) [8], mitochondria [7] or some other signal transduction pathways [17,18]. ER is the site of complex processes such as Ca storage, Ca signaling, processing and folding of newly synthesized membrane and secretory proteins, and triggering of cell response to severe forms of stress, which interfere with ER functions [15]. Cell injury may develop under conditions where ER Ca homeostasis and/or folding or processing of proteins is disturbed (referred to as ER stress), leading to the activation of the unfolded protein response such as suppression of protein synthesis and expression of ER stress-related genes including *XBP1* and *GRP78/BiP* [15].

Recently, a polymorphism of the XBP1 gene was shown to contribute to the genetic risk factor for bipolar disorder [6]. The polymorphism (−116C→G) in the promoter region of XBP1 was significantly more common in Japanese bipolar patients (odds ratio = 4.6) and overtransmitted to affected off-

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Table 1
Basal Ca level and 5-HT-induced Ca response sorted by −116C/G polymorphism of XBP1 gene in Japanese healthy subjects

Ca signaling	Genotype			ANOVA
	C/C (N = 12)	C/G (N = 58)	G/G (N = 47)	
Basal Ca level	63.0 ± 7.8	59.8 ± 2.2	61.4 ± 2.0	F = 0.23, P = 0.79
5-HT-induced Ca response	76.3 ± 8.0	88.5 ± 4.4	85.8 ± 3.6	F = 0.84, P = 0.44

Results are expressed as mean ± S.E.M.

spring in trio samples of the NIMH Bipolar Disorder Genetic Initiative [6]. XBP1-dependent transcription activity of the −116G allele was lower than that of the −116C allele, and in the cells with the G allele, induction of XBP1 expression after ER stress was markedly reduced [6]. Moreover, valproate rescued the impaired response by inducing ATF6, the gene upstream of XBP1 [6]. Darier's disease, an autosomal-dominant skin disorder, which is sometimes accompanied by bipolar disorder, was reported to be caused by mutations in the ER calcium adenosine triphosphatase (Ca-ATPase) gene [5]. In addition, the recent finding that valproate increases expression of the ER stress protein also suggests that the ER plays a role in bipolar disorder [2].

Therefore, it is possible that the altered Ca signaling may be due to the dysfunction of ER stress response by −116G allele of the XBP1 gene. In this study we examined the relationship between the −116C/G polymorphism of the XBP1 gene and the basal Ca level or 5-HT-induced Ca response in the platelets of healthy subjects.

One hundred and seventeen unrelated healthy volunteers were all Japanese recruited from laboratory, office or hospital staff at Hokkaido University. They all underwent a direct interview to exclude psychiatric disorders classified according to DSM-IV. There were 82 males and 35 females, and the average age was 34.1 ± 8.7 (mean ± S.D.) years. They had no physical illness and were all drug free for at least 4 weeks before blood sampling. After complete description of the study, informed consent was obtained from all subjects. The research protocol was approved by the ethics committee of Hokkaido University Graduate School of Medicine.

The isolation of platelets and the measurement of intraplatelet Ca concentration were performed as described previously [9]. Briefly, platelet-rich plasma was incubated with 4 μM fura-2-AM (Dojindo, Kumamoto, Japan), a Ca sensitive fluorescent probe, for 15 min at 37 °C. After centrifugation, the resulting platelet pellet was suspended at 1 × 10^8 cells/ml in Krebs–Ringer HEPES buffer. The samples were prewarmed in a cuvette at 37 °C for 4 min and then 10 μM 5-HT was added to the incubation medium. Fluorescence was measured on a Hitachi F-2000 fluorometer with excitation at 340 and 380 nm, and with emission at 510 nm. We measured both basal Ca concentration and the maximum Ca increase (initial peak-resting level) induced by 10 μM of 5-HT.

DNA was extracted from 20 ml of whole blood by standard methods. For the genotyping of XBP1 gene −116C/G polymorphism, the TaqMan 5′-exonuclease allelic discrimination assay was used. Primers and probes for TaqMan assay were as follows: primers, XBP1-F, CTGTCACTCCGGATGGAAATAAGTC, and XBP1-R, ATCCCTGGCCAAAGGTACTTG, and probes, XBP1-C, VIC-CTCCCGCACGTAAC-MGB, and XBP1-G, FAM-TCCCGCAGGTAAC-MBG. Amplification was performed under the following conditions: 10 min at 95 °C, 40 cycles of 15 s at 92 °C and 1 min at 60 °C in a GeneAmp PCR system 9700 thermocycler. Genotypes were determined using an ABI 7000 HT sequence detection system (Applied Biosystems, Foster City, CA, USA).

The associations between the XBP1 gene polymorphism and, basal Ca level or 5-HT-induced Ca response were assessed by one-way ANOVA.

The basal Ca concentration and 5-HT-stimulated Ca response sorted by XBP1 gene polymorphism are shown in Table 1. Observed genotype distributions were consistent with Hardy–Weinberg equilibrium. The distribution of the XBP1 genotypes in our sample was almost same as in another Japanese sample [6]. There was no significant difference in the basal Ca level or 5-HT-induced Ca mobilization among the healthy subjects with −116C/C, C/G, and G/G genotypes (Table 1).

The following findings expect the possible relationship between the XBP1 gene polymorphism and the Ca signaling abnormality. First, the enhanced Ca response to various agonists in peripheral blood cells is one of a few confirmed biological markers for bipolar disorder [1,3,8,10,11,14,16,19,20]. Second, the dysregulations of Ca homeostasis may be involved in the pathophysiology of bipolar disorder, from the fact that the dysinhibition of Ca mobilization in the presence of PKC inhibitor is observed in bipolar disorder [17] and that agonist-induced Ca responses are enhanced in the presence of myosin light chain kinase inhibitor, which are reversed by treatment with lithium [18]. Third, it is possible that the ER dysfunction may result in abnormal Ca homeostasis. The Ca response to thapsigargin, a ER Ca^{2+}-ATPase inhibitor, are reported to be enhanced in bipolar patients compared to unipolar patients and normal controls [4]. Fourth, the DNA microarray analysis of lymphoblastoid cells derived from pairs of twins discordant with respect to bipolar disorder suggests that the XBP1 polymorphism may contribute to the genetic risk factor for the illness [6]. Last, the expression of GRP78/BiP, one of ER stress proteins, plays a direct and important role in Ca mobilization. It is reported that agonist-stimulated Ca response and thapsigargin-induced Ca release are enhanced in GRP78/BiP-transfected cells compared to control cells [13].

Unexpectedly, the present results did not indicate any significant relationship between XBP1 genotype and basal

Ca concentration or 5-HT-induced Ca mobilization in the platelets of healthy subjects. Since drug-free samples are indispensable to measure intraplatelet Ca concentration [9], in this preliminary study we first examine the association between XBP1 gene polymorphism and Ca signaling in healthy controls. We have already indicated that the range of the 5-HT-stimulated Ca response in normal subjects is widely distributed and fairly overlapped with that in bipolar disorder [11]. Moreover, depressed patients with a Ca response above 280% of basal which corresponds to the value of the mean + 2S.D. for normal controls, exhibit a good response to mood stabilizers [12]. Thus, the higher Ca response depressed patients show, the more probably their diagnosis may be bipolar disorder. This parameter seems to be a continuous marker from normal control to bipolar disorder. On the other hand, the G allele of XBP1 gene, a risk allele for bipolar disorder, is also observed in normal controls, not only in bipolar disorder [6]. Therefore, it is of significance to examine the relationship between the XBP1 genotype and the Ca response in normal subjects. Further studies are necessary to examine the relationship between them in patients with bipolar disorder. Another limitation of this study is to measure Ca response in the mature platelets that lack a nucleus and do not have a part of ER stress response including the XBP1 loop. Using lymphocytes or lymphoblastoid cells as samples may result in the different finding from the present study.

In conclusion, the present study suggests that the XBP1 gene polymorphism is not associated with the basal Ca level or 5-HT-stimulated Ca response in the platelets of healthy control subjects. Further investigations are needed to examine the relationship in the different peripheral blood cells and/or in larger samples from patients with bipolar disorder.

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Age Related Changes in 5-methylcytosine Content in Human Peripheral Leukocytes and Placentas: an HPLC-based Study

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Summary

The goal of the present study was to investigate inter-individual and age-dependent variation of global DNA methylation in human tissues. In this work, we examined 5-methyldeoxycytidine (^{met}C) content by HPLC in human peripheral blood leukocytes obtained from 76 healthy individuals of ages varying from 4 to 94 years (yr), and 39 human placentas from various gestational stages. The HPLC analysis revealed a significant variation of ^{met}C across individuals and is consistent with the previous findings of age-dependent decrease of global methylation levels in human tissues. The age-dependent decrease of ^{met}C was relatively small, but statistically highly significant ($p = 0.0002$) in the aged group (65.9 ± 8.9 [mean age ± SD] yr; n = 22) in comparison to the young adult group (19.3 ± 1.4 yr; n = 21). Males showed a subtle but statistically significant higher mean ^{met}C content than females. In contrast to the peripheral blood samples, DNA extracted from placentas exhibited gestational stage-dependent increase of methylation levels that appeared to inversely correlate with the expression levels of human endogenous retroviruses. These data may be helpful in further studies of DNA methylation, such as inheritance of epigenetic patterns, environment-induced changes, and involvement of epigenetic changes in disease.

Keywords: epigenetics, 5-methylcytosine, HPLC, ageing, leukocytes, placenta, retrotransposons, HERV

Introduction

Cytosine methylation is a reversible epigenetic modification of DNA (Bird, 2002). DNA methylation plays a key role in a number of fundamental biological functions (Li *et al.* 1993; Miura *et al.* 2001; O'Neill *et al.* 1998; Sado *et al.* 2000; Walsh *et al.* 1998). Numerous studies reveal a pathway of gene silencing by DNA methylation through methyl-CpG-binding protein and histone deacetylase multiprotein complexes (Bird & Wolffe, 1999; Razin, 1998). Mammalian genomes show major changes of DNA methylation patterns during development, which include genome-wide demethylation in early embryogenesis followed by global *de novo* methylation (Hsieh, 2000). Mitotic transmission of DNA methylation patterns demonstrates partial stability (Riggs *et al.* 1998). Such patterns may be changed by hormones, nutritional factors, aging, and stochastic events in the cell (Thomassin *et al.* 1983; Wolff *et al.* 1998). For example, age-dependent increase of methylation of some genes was identified in the colorectal mucosa (Ahuja & Issa, 2000). Although traditionally DNA methylation has not been considered to be passed from

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parents to offspring, there is increasing evidence that some of the epigenetic signals exhibit partial meiotic stability and can be transmitted from one generation to another (Morgan *et al.* 1999; Rakyan *et al.* 2002; Roemer *et al.* 1997; Sutherland *et al.* 2000). These observations indicate that epigenetic status, unlike DNA sequences, is a dynamic feature of genes and genomes, and studies investigating variation of epigenetic patterns across individuals are of significant interest. There are several methods to experimentally investigate DNA methylation. Two common approaches, such as methylation-sensitive enzyme-based Southern blot hybridization and the bisulfite modification sequencing method, are most suitable for relatively short and targeted DNA methylation analyses (Rein *et al.* 1998). A high performance liquid chromatography (HPLC)-based analysis does not provide information in terms of specific loci, but rather investigates global DNA methylation levels. A series of global DNA methylation studies were performed in rodents and humans, and such studies indicated that global methylation levels decrease with age (Drinkwater *et al.* 1989; Tawa *et al.* 1990; Wilson & Jones, 1983; Wilson *et al.* 1987). However, human DNA methylation experiments were performed on cultured cells that may exhibit DNA methylation differences in comparison to native cells (Wilson & Jones, 1983; Wilson *et al.* 1987). In addition, only small samples (e.g., n = 8) were tested and the techniques used were not fully informative (e.g. *Hpa* II digestion and end-labelling) (Drinkwater *et al.* 1989; Wilson & Jones, 1983; Wilson *et al.* 1987). To our knowledge, no global DNA methylation studies thus far have attempted to address the question of age dependent DNA methylation changes in the genome. In this work, we perform a detailed HPLC-based analysis of age-related methylation changes in the DNA from human peripheral leukocytes and placenta.

Materials and Methods

Genomic DNA Extraction and Digestion Conditions

Blood samples were obtained from healthy individuals with informed consent. DNA was extracted using the standard proteinase K/phenol method after the lysis of red blood cells in hypotonic ammonium chloride solution. Prior to the conversion of high molecular weight-DNA to mononucleosides, 30 μg of genomic DNA was treated with 880 μg RNase A (Sigma-Aldrich, St. Louis, MO, USA) and 220 units RNase T1 (Roche Diagnostics, Mannheim, Germany) in a total volume of 100 μl for 60 min at 37°C, and ethanol-precipitated at room temperature. This was done because we found that DNA extracted from peripheral leukocytes contained small amounts of RNA and that DNA digests treated with the enzymes produced an RNA-derived peak overlapping with that of ^{met}C in the original procedure employed by Tawa *et al.* (1990) The precipitated DNA was dissolved in 30 μl TE after two rounds of rinsing with 70% ethanol. DNA treatment with nuclease P1 (Roche) and calf intestinal alkaline phosphatase (Takara Shuzo, Shiga, Japan) was carried out as described by Tawa *et al.* (1990) For the analysis of methylation levels in developing placentas, we used samples collected in 1997, except for the term placentas.

HPLC Analysis

Deoxynucleosides, 2′-deoxyadenosine (dA), 2′-deoxycytidine (dC), 2′-deoxyguanosine (dG), thymidine (dT), and 5-methyl-2′-deoxycytidine (^{met}C), were purchased from Sigma-Aldrich (St. Louis, MO, USA). All chemicals were of analytical reagent grade. The HPLC system used consisted of a Waters 2690 separations module (Waters, Milford, MA, USA) equipped with a Waters 2487 dual absorbance detector (Waters). All data were processed with a Millennium 32 chromatography manager (Waters). Separation of the five deoxynucleosides was performed by a column (2.1 × 150 mm) filled with XTerra MS C18 (particle size 3.5 μm, Waters). The column oven temperature was set at 40 °C. The nucleosides were eluted by 20 mM sodium tetraborate, with a flow rate of 0.2 ml/min. We used 20 mM sodium tetraborate instead of 0.1% (v/v) phosphoric acid that was used as a mobile phase by Tawa *et al.*, which, in addition to the aforementioned RNase-pretreatment of DNA, allowed clear separation of ^{met}C from an otherwise overlapping peak of a compound resulting from RNA digests. The UV absorbance of the column eluant was monitored at 273 nm. The injection volume of samples was 20 μl. dC, dG, ^{met}C, dT, and dA were

eluted at the retention time of 5.86, 9.80, 13.51, 16.57 and 40.99 minutes, respectively. The area of each peak was used for the quantitative analysis. Linear calibration curves were obtained in the concentration of a range of 1.0 to 100.0 μM for dC, dG, dT, and dA, and of a range of 0.05 – 5.0 μM for ^{met}C. The correlation coefficient of the curve was 0.999 for every compound. The detection limit of ^{met}C was 0.01 μM (S/N = 3). Day-to-day precision was examined by analyzing the deoxynucleoside standards (10 μM dC, dG, dT, and dA in each, and 0.5 μM ^{met}C) at the start and end of every experiment. All samples were measured in duplicate. Coefficients of variation of the deoxynucleosides between experiments (n = 34: 17 × 2) ranged from 0.89% to 1.55%, except for that of dA, which showed exceptionally large variation (4.07%). The ^{met}C content has usually been expressed as a ratio either to the total DNA or to all deoxycytidines, dC plus ^{met}C. In this work, we represented ^{met}C contents in genomic DNA as relative values of ^{met}C to the sum of dC and ^{met}C. Statistical analyses were performed using the StatView software (SAS Institute Inc., Cary, NC, USA). Difference of the means between two groups was evaluated by the Mann-Whitney's U test and was considered significant when the p value was less than 0.05.

Human Endogenous Retrovirus (HERV) Expression Analysis

HERV expression levels were examined by slot blot hybridization in three placentas in each trimester (7-week, 15 and 16-week, and 32-week gestations that correspond to the first, second, and third trimester, respectively). DNase-treated RNA (6 μg) was blotted onto a nylon membrane in duplicate. DNase and RNase-treated RNA was simultaneously blotted and was confirmed to give no signal. The blots were prehybridized at 68°C for 1 hr and then hybridized with random-primed DNA probes at 68°C for 1 hr in ExpressHyb solution (Clontech, Palo Alto, CA, USA). Probe DNAs were prepared by PCR amplifications. Primer sequences and PCR conditions are as follows: (HERV-K primer set) 5′-GTAAGCGGGATGTCACTCAG and 5′-TTCCTACAACTAGCATATAAGG, (HERV-E primer set) 5′-GCTCCTGAYATAATTCCART and 5′-CCACTCATYATCTTTCCARTT; 30 cycles of denaturing at 94°C for 10 sec, annealing at 51°C for 15 sec, and extension at 72°C for 30 sec using 20 ng of genomic DNA in 15 μl of the standard reaction mixture. PCR products of 589 bp (HERV-K) and 468 bp (HERV-E) were confirmed by direct sequencing to be heterogeneous collections of the same families. To calibrate the loading amounts of RNA, the stripped membranes were rehybridized with a β-actin probe. Final washing conditions of the membranes were twice with 0.1 × SSC/0.1% SDS for 10 min at 52°C (HERV-K) and 53°C (HERV-E and β-actin). Signal intensities were measured with a BAS 2000 imaging analyzer (Fujix, Tokyo, Japan) after exposure of the membranes overnight (HERVs) or for 1 hr (β-actin). Transcription levels of HERVs were adjusted with those of β-actin.

Genotype Analyses

The methylenetetrahydrofolate reductase (*MTHFR*) C677T and A1298C single nucleotide polymorphisms were determined by methods described elsewhere (Friedman *et al.* 1999; Frosst *et al.* 1995). Differences in the distribution of ^{met}C contents in the three genotype-groups were evaluated by the Kruskal-Wallis' test.

Results

Age-Dependent Change of the ^{met}C Content in Peripheral Leukocytes

We examined DNA methylation levels in peripheral leukocytes obtained from 76 healthy individuals aged from 4 to 94 years (mean ± SD: 33.2 ± 22.7 yr). Relative contents of ^{met}C to those of dC and ^{met}C were between 3.84% and 4.12% (mean ± SD: 3.99% ± 0.07%). ^{met}C density demonstrated age-dependent decrease (Fig. 1). We arbitrarily classified the tested individuals into 4 groups: juvenile group (mean age, 10.9 ± 2.6 yr; n = 18), young adult group (19.3 ± 1.4 yr; n = 21), older adult group (31.3 ± 5.6 yr; n = 15), and aged group (65.9 ± 8.9 yr; n = 22). The aged group showed the lowest mean ^{met}C content among the four groups (Table 1). The loss of the mean ^{met}C content in the aged group was 2% of the mean ^{met}C content in the young adult group, which exhibited the highest ^{met}C density, and this difference was statistically highly significant

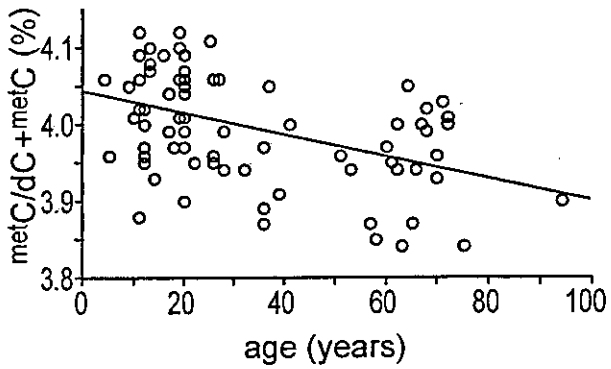

Figure 1 Change in methylation levels with age in human leukocytes. 5-methyldeoxycytidine contents of 76 healthy donors were represented as relative ratios (%) to the sum of deoxycytidine (dC) plus 5-methyldeoxycytidine (^{met}C) and were plotted against the age of donors. Linear regression analysis provided a slope of –0.001 (standard error, $3.225e^{-4}$) and a correlation coefficient of $r = -0.455$, n = 76.

($p = 0.0002$). Females (n = 44; 35.9 ± 24.7 yr [mean age ±SD]; $^{met}C/(dC + {}^{met}C) = 3.975 \pm 0.067$) showed lower methylation level than males (n = 32; mean age, 29.3 ± 19.5 yr; $^{met}C/(dC + {}^{met}C) = 4.019 \pm 0.069$) ($p = 0.0067$). It is also important to note that the inter-individual difference of subjects with the same age (20 yr; n = 12) between the highest and lowest ^{met}C contents reached up to 4.7% of the mean ^{met}C content in this age group (4.03 ± 0.06).

The MTHFR C677T and A1298C Genotype Analyses

The C677T genotype was determined in all 76 individuals. The allele frequencies of 677C and 677T were 0.72 and 0.28, respectively. Homozygosity of 677T was found in seven individuals, and interestingly all were in the older age groups (4 in the older adult group and 3 in the aged group). The difference of mean ^{met}C content between the young adult and the aged groups did not change and was still significant when the individuals with the TT genotype were excluded (4.030 ± 0.003 in the younger adult group vs 3.947 ± 0.004 in the aged group; $p = 0.0002$). The mean ^{met}C contents (and SD) in individuals with the CC (n = 13), CT (n = 17), and TT (n = 7) genotypes in the combined older adult and aged groups were 3.954 (±0.064), 3.952 (±0.057), and 3.990 (±0.089), respectively, and no statistically significant difference in the distribution of ^{met}C contents in the three genotype-groups was detected ($p = 0.42$). The allele frequencies of 1298A and 1298C were 0.85 and 0.15, respectively. Because of the small number of CC homozygotes (n = 3) and deviation of age distribution in the three genotypes, a statistical analysis for the effect of the A1298C polymorphism on ^{met}C contents was not performed.

Gestational Age-Dependent Change of Methylation in Placentas

Compared to embryonic tissues, the placenta exhibits unique features of epigenetic DNA regulation including X inactivation (Looijenga *et al.* 1999; Uehara *et al.* 2000), genomic imprinting (Higashimoto *et al.* 2002; Jinno *et al.* 1995), and the expression of retrotransposons (Kjellman *et al.*1999; Mi *et al.* 2000). We were interested in the density of ^{met}C changes in the developing

Table 1 Methylcytosine content in genomic DNA of human peripheral leukocytes and placentas

Tissue*1	Group	Age (years) or gestation (weeks)*2	No. of samples	$^{met}C/dC+{}^{met}C$ (%)*3	p value
PBL	Juvenile	4–14 (11)	18	4.018 ± 0.066	
	Young adult	16–22 (19)	21	4.030 ± 0.056	
	Older adult	25–41 (31)	15	3.977 ± 0.068	0.0002
	Aged	51–94 (66)	22	3.948 ± 0.064	
Placenta	First trimester	6–13 (9)	15	2.850 ± 0.080	
	Second trimester	14–23 (18)	12	3.004 ± 0.098	>0.0001
	Third trimester	32–40 (38)	12	3.123 ± 0.082	

*1: PBL, peripheral blood leukocytes.
*2: means in parentheses.
*3: Means and SD are shown.

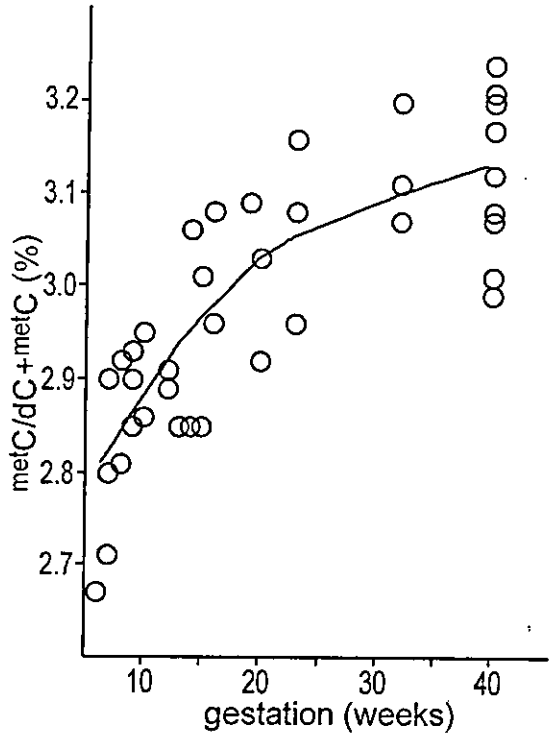

Figure 2 Change in methylation levels in developing placentas. A total of 39 human placentas with various gestational ages (6 to 40 weeks) were analyzed. Relative ^{met}C contents were plotted against the gestational age as described in Fig. 1. A curve fit line was drawn by the Lowess (locally weighted scatterplot smoother) method (tension = 66).

placentas and investigated methylation levels of 39 placentas at various gestational stages (Table 1). As shown in Fig. 2, ^{met}C contents increased as the gestational age advanced. The pattern of increasing ^{met}C density seemed to consist of two phases: a rapid increase during the first half of pregnancy, followed by a more moderate increase until term. ^{met}C contents increased on average by 10% in placental DNAs in the first trimester until term, but such levels only reached on average ~78% methylation of the leukocyte DNA. When methylation levels in the placenta were compared with those in the liver and brain of 5 fetal specimens with gestation of 15 to 20 weeks, the mean value of relative ^{met}C contents in the placenta was approximately 80% of that in the brain and liver (Table 2). Thus, this lower DNA methylation is a feature inherent to the placenta and is not attributed to the difference of sampling before and after birth.

HERV Expression in Developing Placentas

To investigate the biological role of the increasing methylation levels in the placenta, we examined expression levels of HERV-K and HERV-E in developing placentas by RNA slot blot hybridization. HERV-K loci are strictly suppressed and Northern blot cannot detect any clear signals in normal tissues (Sugimoto *et al.* 2001). Among HERV-E loci, *ERVE1* is abundantly expressed in thyroid and pancreas, and weakly in placenta (Shiroma *et al.* 2001). Thus, signals detected by slot blot hybridization are likely to represent the sum of 'leaky' transcripts from various loci of the HERV-K and HERV-E families. The level of HERV expression in the late stage placenta was suppressed by 10 to 20% of that in the earlier stage placenta (Fig. 3). Thus, gross levels of both HERV transcripts show an inverse correlation with the change of methylation levels.

Discussion

Research on DNA methylation has been rapidly developing over the last decade and has demonstrated the involvement of epigenetic mechanisms in a wide range of normal and pathological cellular phenomena. Age related changes in DNA modification are of particular interest since they may aid understanding of the molecular basis of various non-Mendelian features of complex disease, including relatively age of onset (Petronis, 2001). It is known that global DNA

Table 2 Methylcytosine content in fetal placenta, brain, and liver DNA

Tissue	Case 1 (15w)	Case 2 (16w)	Case 3 (19w)	Case 4 (20w)	Case 5 (20w)	Mean ± SD
Placenta	2.85	3.08	3.09	2.92	3.03	2.994 ± 0.105*
Brain	3.95	3.81	3.84	3.81	3.80	3.842 ± 0.062
Liver	3.74	3.59	3.59	3.68	3.91	3.702 ± 0.133

Methylcytosine contents are shown as relative ratios to the sum of deoxycytidine and methyldeoxycytidine (%). *$p < 0.0001$ when compared with the mean of brain or liver.

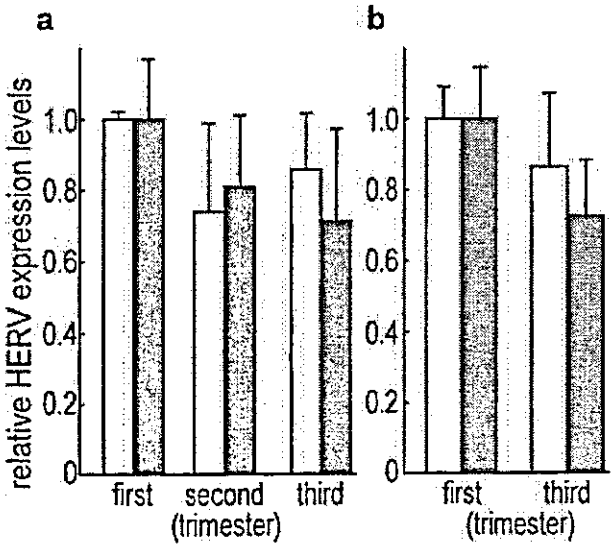

Figure 3 Change in transcription levels of human endogenous retroviruses, HERV-K (a) and HERV-E (b), in developing placentas. Signal intensities of HERV transcripts were measured in duplicate by BAS 2000 (Fujix) and adjusted by those of β-actin. Mean values of the corrected transcription levels of the HERVs were obtained in two separate experiments from 3 placental specimens in each categorized gestation group. In addition, each value was represented as a relative ratio to the mean value of the first trimester group in each experiment in order to allow the direct comparison of those values in two independent experiments. The means and SDs of 3 samples in each are depicted with boxes and bars where open, and filled boxes indicate results obtained from experiment 1 and experiment 2, respectively.

methylation levels decline with age in human and mouse, but it has not been accurately documented to what degree change throughout the life span. In addition, thus far no global HPLC-based DNA methylation study has attempted to address the question of age-dependent DNA methylation changes in native peripheral leukocytes, which are the most available cells for human studies. In the present study, we used HPLC to characterize the dynamics of the global DNA methylation levels in human peripheral leukocytes, and placental DNA, from individuals of different age groups.

One of the main findings of the above study is evidence for inter-individual variation of global DNA methylation levels. At least to some extent such variation is related to aging, a finding which is consistent with other similar studies on cultured fibroblasts and epithelial cells, and purified T cells (Golbus *et al.* 1990; Wilson & Jones, 1983; Wilson *et al.* 1987). In contrast to previous studies that demonstrated a larger age-dependent decrease of global methylation levels, for example a slope of –0.004%/year in the linear regression analysis and about 10% loss of methylcytosine in 50 years (Drinkwater *et al.* 1989; Wilson *et al.* 1987), the age dependent decrease in density of ^{met}C was much smaller, and according to our data the slope was only −0.001%/year and 2% loss in approximately 50 years. All this may have resulted from the differences in methods used, as well as DNA methylation differences in cultured cells (e.g. 2.37–3.00% in foreskin fibroblast culture; Wilson & Jones, 1983) versus native cells (3.84–4.12%; the current study). Loss of ^{met}C content in the aged group (mean age 66 yr; n = 22) was on average 2% of the mean ^{met}C content in the young adult (mean age 19 yr; n = 21). However, the difference was statistically highly significant (p = 0.0002). These results lead to two conclusions. Firstly, global DNA methylation levels are fairly stable in human peripheral leukocytes throughout the lifespan. Secondly, even small differences can be reliably detected by HPLC analysis.

The present study also detected a subtle but statistically significant difference (p = 0.0067) between males and females. The higher, albeit subtle, global methylation level in males compared to females was a somewhat surprising result considering the presence of an inactivated X chromosome in females, a phenomenon that is known to be mediated by DNA hypermethylation of the X chromosome. On the other hand, an HPLC-based DNA methylation analysis detected prolactin treatment-induced hypomethylation of rat liver and kidney DNA samples (Reddy & Reddy, 1990). These findings may indicate that hormones can be internal 'environmental' factors that could influence DNA methylation states. Alternatively, it is also possible that the difference between males and females may be a reflection of mean age difference of the two populations analyzed (males, 29.3 ± 19.5 yr; females, 35.9 ± 24.7 yr). However, the difference between males and females remained to be statistically significant (p = 0.0159) when the comparison of ^{met}C density was performed in individuals in a limited age range of 11 to 41 years (4.035 ± 0.063 in males, mean age 22.3 ± 8.8 yr; n = 24, vs 3.989 ± 0.065 in females, mean age 19.6 ± 7.5 yr; n = 26). This finding is consistent with the result of the study by Golbus *et al.* (1990) that showed a lower ^{met}C

content in women than in men, albeit statistically not significant.

Another finding that was not clearly indicated in any previous studies is the presence of a noticeably large difference of global methylation levels in individuals of the same age. It would be of significant interest to find out if the observed variation of the ^{met}C content between individuals with the same age is genetically determined. In this regard, it is intriguing that the homozygous 677TT genotype of the methylenetetrahydrofolate reductase (*MTHFR*) gene was shown to correlate with a diminished level of global DNA methylation compared with the 677CC genotype in patients with coronary atherosclerosis or other heart diseases (Friso *et al.* 2002). In our analysis of healthy subjects, the C677T polymorphism did not seem to cause a significant difference in the ^{met}C content. This may partly be attributed to differences in nutritional status between healthy subjects and clinical patients, as well as other genes coding for enzymes involved in methyl metabolism such as methionine synthase (Paz *et al.* 2002). In addition, many factors including methyl binding proteins, heterochromatin binding proteins, histone acetylases/deacetylases, and histone methyltransferases, as well as DNA methyltransferases, have been suggested to be involved in the formation and maintenance of DNA methylation patterns (Ben-Porath & Cedar, 2001).

In contrast to the change of methylation levels in peripheral leukocytes, the ^{met}C content in placentas drastically increased with gestational age. Our HPLC analysis detected the dynamic change of global DNA methylation in placenta. The mean ^{met}C content in the term placentas was lower than that of peripheral leukocytes in any age group. It is known that the placenta is one of the tissues with lower methylation levels of the genome, and that the DNA fraction exhibiting the lowest ^{met}C content is the Eco family of tandemly repeated sequences of the alphoid type (Gama-Sosa *et al.* 1983). To our knowledge, this study is the first to demonstrate changes of global methylation levels of developing placentas in humans. We have to identify DNA sequences that are progressively methylated, as well as whether and how the global hypomethylation contributes to placenta-specific features regarding X inactivation, genomic imprinting, and control of retrotransposon activity (Higashimoto *et al.* 2002; Jinno *et al.* 1995; Kjellman *et al.* 1999; Looijenga *et al.* 1999; Mi *et al.* 2000; Uehara *et al.* 2000). In our analyses, the global methylation levels of placentas seemed to be inversely correlated with the gross levels of HERV-K and HERV-E transcription, which supports the idea that DNA methylation is a mechanism that reduces transcriptional activity of retro-elements (Bird, 1995; Yoder *et al.* 1997).

It is important to determine in which DNA fractions the age-dependent decrease in DNA methylation is taking place, in order to better understand the role of DNA methylation in age-related physiological and pathological changes. The apparent decrease of the ^{met}C content is a net effect of increase and decrease of methylation (Dunn, 2003). The age-dependent methylation change in CpG islands seems infrequent (Tra *et al.* 2002). In contrast, the age-dependent demethylation of repetitive sequences such as satellite DNAs was frequently observed in humana and other animals (Hornsby *et al.* 1992; Howlett *et al.* 1989; Suzuki *et al.* 2002). While repetitive sequences may be major constituents contributing to the age-related decrease of methylation, ribosomal DNA repeats were hypermethylated with age in rat liver (Oakes *et al.* 2003), and mutations in the *ATRX* gene showed opposite effects on methylation of repetitive sequences depending on the repeat types (Gibbons *et al.* 2000). Elucidation of DNA methylation changes with age at the individual locus level is far from complete, and beyond the scope of our present study.

In conclusion, we demonstrated the usefulness of HPLC for the population study of global DNA methylation. Our data may be helpful in experimental designs for population studies exploring various aspects of DNA methylation, such as inheritance of epigenetic patterns, environment-induced changes, and involvement of epigenetic factors in human diseases.

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Title: Increased and decreased cortical reactivities in novelty seeking and persistence: a multichannel near-infrared spectroscopy study in healthy subjects

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Near-infrared spectroscopy; Personality; Novelty seeking; Persistence; Cerebral blood flow; Bedside monitoring

Abstract

Background: Near-infrared spectroscopy (NIRS) has enabled completely noninvasive measurements of regional cerebral blood volume (rCBV) changes in cortices. In the present study, we investigated the relationships between rCBV changes assessed with NIRS and two dimensions of personality, novelty seeking and persistence.
Methods: Thirty right-handed healthy volunteers participated in the study. Their personality traits were assessed using the Temperament and Character Inventory (TCI), and changes in oxy- and deoxy-hemoglobin concentrations were monitored during 40s unilateral finger tapping tasks over the subjects' bilateral temporal regions using a 24-channel NIRS machine.
Results: The oxy-hemoglobin concentration increases were significantly correlated positively with novelty seeking scores and negatively with persistence scores in the TCI during the initial time segment of the left-finger tapping task.
Conclusion: Increased and decreased brain activations demonstrated using multichannel NIRS were assumed to characterize the cortical reactivities underlying novelty seeking and persistence temperament, respectively.

Introduction

Personality is defined as the ingrained patterns of thought, feeling, and behavior characterizing an individual's unique lifestyle and mode of adaptation, and resulting from constitutional factors, development, and social experience. It has traditionally been conceptualized as consisting of several factors or dimensions. One of the major models of personality was proposed by Cloninger et al. [1] on the basis of the hypothesis that personality consists of two components, biological and social, which are often

called "temperament" and "character", respectively. Temperament is defined as biologically based, heritable, and stable throughout life, and character as socially acquired and refers to individual differences in voluntary goals and values [2]. Scores in four temperament dimensions and three character dimensions are defined using the Temperament and Character Inventory (TCI).

The neurobiological substrates of personality, especially these of temperament, have been studied from three points of views: psychological functions, neurochemical substances, and brain function characteristics responsible for each dimension of personality. For example, Cloninger [3] proposed four dimensions of temperament, that is, novelty seeking, harm avoidance, reward dependence, and persistence, and assumed behavioral activation, behavioral inhibition, behavioral dependence, and behavioral persistence systems as their psychological correlates, respectively. He also hypothesized three monoamine transmitters, namely, dopamine, serotonin, and noradrenaline, as the possible neurochemical substrates of the former three dimensions of temperament, respectively.

Brain function characteristics responsible for personality dimensions have been most proposed for the introversion-extraversion dimension, which is included in most major current models of personality as one of the fundamental dimensions. Introversion and extraversion were hypothesized to represent higher and lower cortical activities, respectively, especially in the frontal lobes, due to overactive and underactive reticulo-thalamo-cortical pathways [4] or to higher and lower activities in the behavioral inhibition system consisting of the ascending reticular activating system, the frontal lobe, septal regions, and hippocampus [5]. Recent advances in functional brain imaging methodologies have enabled the direct examination of these hypotheses in living human brains. Extraversion scores in the Neuroticism, Extraversion and Other-Five-Factor Inventory were negatively correlated with regional cerebral blood flow (rCBF) in the frontal lobes, although positively in the temporal lobes and limbic regions, in a [^{15}O]H_2O-PET study [6]. Extraversion-introversion scores in the Eysenck Personality Inventory were also negatively correlated with rCBF in all the brain regions in female subjects in a ^{133}xenon inhalation study [7]. The results were replicated in another study as lower rCBF in the temporal lobes in extraverts [8]. The results of these three studies support the hypothesis of higher and lower cortical activities in introversion and extraversion, respectively.

As for the temperament dimensions in the TCI, functional brain imaging studies demonstrated significant associations between brain activation and novelty seeking scores. Novelty seeking scores were positively correlated with glucose metabolism in the right middle frontal gyrus but were negatively correlated with glucose metabolism in the right middle temporal gyrus, left precentral gyrus, left parahippocampal gyrus and substantia nigra in a [^{18}F]-fluorodeoxyglucose (FDG) positron emission tomography (PET) study [9]. Novelty seeking scores were positively correlated with rCBF in the right insula and left anterior cingulate in a single photon emission computed tomography (SPECT) study [10]. There has been only one study that examined the relationships between all the seven personality dimensions in the TCI and rCBF [11]. The SPECT study reported many significant relationships in male subjects: novelty seeking with activation in the left precentral and postcentral gyrus and deactivation in the temporal gyri, occipital lobe, and precuneus; persistence with activation in the temporal , parietal, occipital, and limbic lobes and deactivation in the parietal, temporal, frontal lobes, the rolandic operculum, and insula; and self-directedness with activation in the left frontal lobe and deactivation in the precentral

gyrus, frontal lobe, temporal lobe, and occipital lobe mainly on the right side. These results are in partial agreement with those examining the relationships between a few personality dimensions and rCBF or cerebral glucose metabolism.

The functional brain imaging results mentioned above suggest that brain function characteristics, particularly those in cortical activity levels, may serve as neurobiological substrates for introversion-extraversion and novelty seeking dimensions in temperament. However, it should be cautioned that all the six studies measured rCBF or cerebral glucose metabolism while the subjects were in the resting state: that is, the obtained data corresponded to the state of the brain when no activation was demanded. Correlations of introversion-extraversion and novelty seeking scores with brain activities could be much higher if activational changes in rCBF or cerebral glucose metabolism are employed instead of those in the resting state because introversion-extraversion and novelty seeking dimensions refer to the personality characteristics particularly observed in response to environmental stimuli. Among four temperament dimensions in the TCI, novelty seeking and persistence scores are expected to exhibit close relationships with brain activation because they are assumed to represent activation and persistence of behavior; that is, novelty seeking as behavioral activation could correspond to enhanced reactivity of cerebral activities and persistence as behavioral persistence to their reduced reactivity.

The noninvasive and continuous monitoring of such activational changes of brain functions has become possible with the development of near-infrared spectroscopy (NIRS) technology. The successful trials of measuring brain functions in humans using an NIRS oxygen monitor were reported in 1993 by four research groups [12-15], and the spatiotemporal patterns of brain functional changes were demonstrated in 1995 by mapping the topograms of hemoglobin concentration changes using multichannel-NIRS machines [16]. The technological basis of NIRS was reviewed by Koizumi et al. [17], Strangman et al. [18], and Obrig and Villringer [19]. NIRS has a number of advantages: high time resolution, noninvasiveness [20], high tolerance for motion artifacts, portability, and low running cost. All these advantages of NIRS allow the measurement of a brain function along a detailed time course while the brain is activated in a natural state. The brain substrates of emotion have been examined by NIRS [21].

Hemoglobin concentration measured using NIRS is interpreted to indicate regional cerebral blood volume (rCBV). Neural activity in the brain causes increases in oxygen consumption and glucose metabolism in the brain tissue, which are followed by an excessive increase in rCBF [22]. The excessive increase in rCBF results in an increase in oxygenated hemoglobin concentration ([oxy-Hb]) and a decrease in deoxygenated hemoglobin concentration ([deoxy-Hb]) during neural activation. An increase in [oxy-Hb] and a decrease in [deoxy-Hb] were demonstrated by NIRS to positively and negatively correlate with rCBF respectively in an $^{15}H_2O$ PET study [23].

In the present study, we monitored cortical reactivities as the [oxy-Hb] and [deoxy-Hb] changes using a multichannel NIRS machine during a finger tapping task in healthy subjects. We then examined the relationships between [oxy-Hb] and [deoxy-Hb] changes and personality features assessed using TCI. A simple motor task rather than a complex cognitive task was employed for brain activation for an easier interpretation of the obtained results from the viewpoint of behavior, on which four temperament dimensions of TCI are based. Such a simple motor task is assumed to be more directly related to the

behavioral activation system (novelty seeking dimension) and behavioral persistence system (persistence dimension) than to the behavioral inhibition system (harm avoidance dimension) and behavioral dependence system (reward dependence dimension): larger [Hb] changes reflect the excitability of neuronal activities and an hence enhanced reactivity of brain functions, and are expected in the subjects with high scores in novelty seeking (strong behavioral activations); smaller [Hb] changes reflect the unchangeability of neuronal activities and hence a reduced reactivity of brain functions and are expected in the subjects with high scores in persistence (strong behavioral persistence). Task performances were also incorporated into the data analysis because [oxy-Hb] change magnitudes in NIRS were demonstrated to depend on task performances in healthy subjects in a multichannel NIRS study, with smaller [oxy-Hb] increases in high performers [24].

Our working hypotheses prior to this study were as follows: 1) significant correlations with [oxy-Hb] and [deoxy-Hb] changes would be obtained positively for novelty seeking scores and negatively for persistence scores in the TCI because these two dimensions are related to the reactivity of brain functions; 2) these correlations would be stronger than those for three dimensions of character and even for the other two dimensions of temperament because character dimensions are assumed to be less biologically based than temperament.

Methods

Subjects

Thirty healthy volunteers participated in this study (age: mean, 27.1 years [SD 3.0]; range, 22-33 years; 15 males and 15 females). None of these volunteers had a medical history of psychoneurological illness, chronic somatic illness, substance abuse or serious head injury, or were receiving any medications. All the subjects were right-handed and gave their written informed consent prior to the study. The present study was approved by the Institutional Research Board of Gunma University Graduate School of Medicine.

Activation tasks

CBV was measured during a finger tapping task. The subjects were required to perform unilateral finger tapping as quickly and precisely as they could during the task periods. The subjects sat on a comfortable chair placing their arms on the armrest in a well-lighted room. The subjects were instructed to look ahead blankly, and to avoid any movements other than the finger tapping. They rehearsed the finger tapping before starting the measurement to ensure that they comprehended the task instructions.

The task consisted of three cycles of 40-s unilateral finger tapping and a subsequent 30-s rest. The rather long task period of 40s was selected on the basis of our preliminary study that showed that the task periods of 20 and 30s usually employed in most NIRS studies were not sufficiently long to reveal the temporal characteristics of hemoglobin concentration changes during the task.

The order of right- and left-finger tapping tasks was counterbalanced among the subjects. The subjects were allowed a 3-min rest between finger tapping tasks on each side. The finger tapping