possessed the uncommon I425V mutation in the coding region of the SERT gene. One study employing complete sequencing of the SERT gene using DNA from 22 individuals with OCD plus controls revealed no variants.[27] A recent report screening for low frequency of SERT gene sequence variations using SNPs found that the I425V mutation was rare, occurring in only one of the 450 individuals;[14] no clinical or demographic data are available on these individuals.[28]

The I425V mutation is located in transmembrane region 8 of SERT (Figure 2), where it is in position to alter the secondary structure of this hydrophobic domain. Its presence locks the mutant transporter in an activated state that is normally achieved in the I425 transporter through the action of nitric oxide and cGMP. Evidence for constitutive activation of SERT by the 425V mutation in transfected human cell lines is presented elsewhere.[14] Interestingly, in the two families we have studied, the I425V variant is on the same DNA strand with the L allele of 5-HTTLPR, a locus found at a distance of >2 kb from I425V. Furthermore, the homozygous 5-HTTLPR LL genotype is present both in the brothers A1 and A2 who have OCD plus AS and in the sisters B1 and B2 who have OCD or OC personality disorder plus AN. (Figure 1). The LL variant leads to two- to three-fold greater transcriptional activity (increased mRNA, SERT sites and serotonin uptake) plus greater response to cyclic AMP and protein kinase C than the SS form.13 Thus, this combination of SERT genotypes represents a unique 'double-hit' with functional consequences in the same, greater transport direction derived from both the coding region I425 mutation and the LL promoter region variant. The combined locked-on, gain-of-function effects of these SERT gene loci may cause an attendant deficit in the potential for adaptive responses to environmental challenges occurring either during early development or in adulthood.

In conclusion, we have found an unusual clustering of multiple, uncommon neuropsychiatric disorders (0.02–2% of the general population for OCD, AN, AS/autism)[2,18,25] associated with an uncommon functional missense variant in the serotonin transporter gene. Individuals with Val425 in two families had neuropsychiatric disorders long hypothesized to have a serotonergic etiological component. The unusual severity and treatment resistance of the disorders in the probands and their siblings likely represent the potent gain-of-function effect of Val425 on serotonin transporter function and may also reflect the combined 'double-hit' effect of Val425 with the more highly transcribed allele of the serotonin promoter polymorphism.

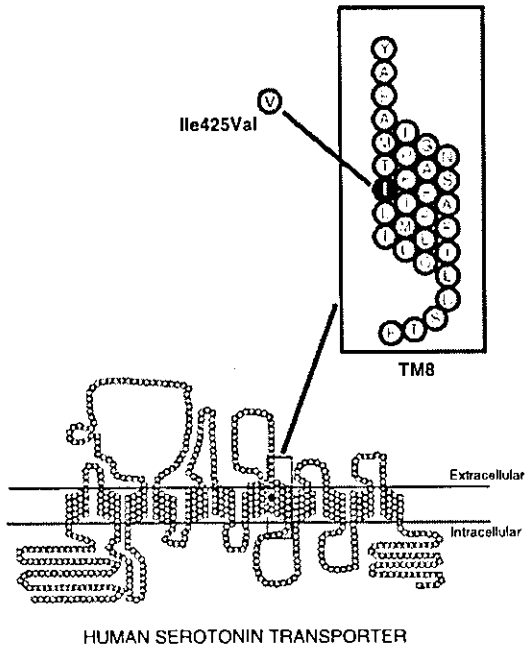

Figure 2 Topological location of the I425V mutation in transmembrane region 8 of the human serotonin transporter.

Methods

Subjects

All patients and healthy controls gave informed consent prior to participating in human research protocols approved by the NIMH-IRP and the University of Pittsburgh School of Medicine Institutional Review Boards. Subjects were psychiatrically evaluated using the Structured Clinical Interview (SCID) for DSM–III–R and DSM-IV and/or Schedule for Affective Disorders and Schizophrenia (SADS) inventory prior to genotyping, as part of ongoing genetic studies of these disorders[3,29–31] (except the CEPH controls and exceptions noted). Healthy controls had no history of any DSM Axis I psychiatric conditions.

SSCP-PCR, detection of the amino acid substitution by modified PCR and 5-HTTLPR genotyping

SSCP-PCR analysis was carried out according to procedures previously described,[31] using 13 primer pairs that cover the entire SERT gene coding sequence (available from authors). A modified primer (5'GAT TAA CAT CAG AAA GAA GAT GC3') and the upper primer of exon 9 were used to introduce a base substitution (T to C) adjacent to the codon of interest, creating an artificial restriction site with only one allelic form.[32] Following amplification of DNA, the PCR product was digested by *Hpa*II yielding two fragments in the case of the rare allele Val425; the product remains intact in the case of the common allele, Ile425. All members of Family A and B were also genotyped for 5-HTTLPR using a modification of the genotyping procedure as previously described.[13]

Acknowledgements

We thank our patient and family collaborators, Drs Gabriella Cora-Locatelli, Margaret Altemus, Theresa Pigott, Catherine Stayer, Joshua Rosenthal plus Diane Kazuba, Lucy Justement, Su-Jan Huang, Liz Maxwell, Julie Guroff, Teresa Tolliver and Theresa B DeGuzman for their contributions to this study, and Drs Klaus-Peter Lesch, Judith Rapoport and Sevilla Detera-Wadleigh for helpful suggestions with the manuscript. During the early stages of this work, Dr Norio Ozaki was supported at the NIAAA and NIMH Intramural Programs by Research Grants (10670923, 13470198, 13877152) from the Ministry of Science Education and Culture of Japan, the Research Grant for Nervous and Mental Disorders from the Ministry of Health and Welfare of Japan, and Special Coordination Funds for Promoting Science and Technology Target-oriented Brain Science Research Program from the Ministry of Science and Technology of Japan.

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Received 29 January 2003; revised 7 April 2003; accepted 16 April 2003

nature genetics

Impaired feedback regulation of XBP1 as a genetic risk factor for bipolar disorder

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The pathophysiology of bipolar disorder is still unclear, although family, twin and linkage studies implicate genetic factors[1]. Here we identified *XBP1*, a pivotal gene in the endoplasmic reticulum (ER) stress response, as contributing to the genetic risk factor for bipolar disorder. Using DNA microarray analysis of lymphoblastoid cells derived from two pairs of twins discordant with respect to the illness, we found downregulated expression of genes related to ER stress response in both affected twins. A polymorphism (–116C→G) in the promoter region of *XBP1*, affecting the putative binding site of XBP1, was significantly more common in Japanese patients (odds ratio = 4.6) and overtransmitted to affected offspring in trio samples of the NIMH Bipolar Disorder Genetics Initiative. 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. Valproate, one of three mood stabilizers, rescued the impaired response by inducing *ATF6*, the gene upstream of *XBP1*. These results indicate that the –116C→G polymorphism in *XBP1* causes an impairment of its positive feedback system and increases the risk of bipolar disorder.

Bipolar disorder is a severe mental illness characterized by recurrent episodes of mania and depression that affects about 1% of the population and is life-threatening as affected individuals may commit suicide. Although the concordance rate in monozygotic twins (>65%) is much higher than in dizygotic twins (>14%), some monozygotic twins are discordant with respect to bipolar disorder[2]. Lymphoblastoid cells of individuals with bipolar disorder have altered signal transduction systems[3–5]. To detect causative genes effectively, we used DNA microarray analysis using lymphoblastoid cells from the twins discordant with respect to bipolar disorder. The overall difference in the gene expression patterns between the discordant twins was more prominent than between control twins (**Supplementary Fig. 1** online). Among the genes downregulated in both affected twins (**Supplementary Table 1** online), we focused on *XBP1* and *HSPA5* (also called *GRP78* and *BiP*), in which we confirmed differential expression by real-time quantitative RT–PCR (**Supplementary Table 2** online). *HSPA5* gene expression is induced by the mood stabilizer valproate[6–8], is regulated by XBP1 (ref. 9) and is located on 22q12, which previously has been linked with bipolar disorder[10–12]. Both genes are essential in ER stress response signaling.

The ER is a protein folding system. When unfolded proteins accumulate in the ER, ER chaperons, such as HSPA5, assist in refolding them[13]. 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 elements, such as *XBP1* and *HSPA5* (refs. 14,15). In parallel with ATF6 protein cleavage, IRE1 proteins on the ER membrane dimerize by dissociation of HSPA5 and subsequently splice *XBP1* mRNA[9]. The spliced mRNA encodes an active form of XBP1 that strongly induces the expression of chaperones, including *HSPA5*, as well as *XBP1* itself[9]. This sequential response, referred to as ER stress response, is elicited *in vitro* by thapsigargin, an inhibitor of ER Ca^{2+}-ATPase[16].

The biological basis of discordance between monozygotic twins is not completely known, but several mechanisms have been identified in some cases, including point mutations, extension of triplet repeat, chromosomal aberrations, altered X-chromosome inactivation and aberrant DNA methylation[17]. Because we did not detect differential expression for *ATF6*, the expression changes of *XBP1* and *HSPA5* could be explained by chromosomal deletion, mutation or different methylation status of *XBP1*. We found, however, no difference of copy number, genomic sequence or methylation status of the upstream region between the twins (data not shown). This finding is well explained by the method we used to choose candidate genes, namely, selecting commonly altered genes in two discordant twins. By this method, we could choose genes in the final common pathway rather than the gene having different sequence or methylation status between discordant twins. Although the primary abnormality causing the discordance is also of interest, the final common pathway should be important in the study of a complex disorder.

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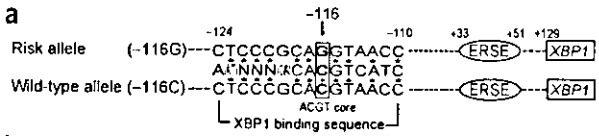
Published online 31 August 2003; doi:10.1038/ng1235



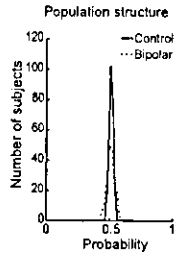
b

Case-control study

Genotype	C/C	C/G	G/G	
Control	65 (0.15)	195 (0.43)	191 (0.42)	
Bipolar disorder	7 (0.04)	99 (0.51)	91 (0.45)	$P = 0.00026$
BPI	6 (0.04)	73 (0.53)	61 (0.43)	$P = 0.0040$
BPII	1 (0.02)	26 (0.46)	30 (0.52)	$P = 0.023$

Allele	C	G	
Control	325 (0.36)	577 (0.64)	
Bipolar disorder	113 (0.29)	281 (0.71)	$P = 0.010$
BPI	85 (0.31)	195 (0.69)	$P = 0.081$
BPII	28 (0.25)	86 (0.75)	$P = 0.015$

c



d

Transmission disequilibrium test of the G allele

	Transmitted	Not transmitted	Ratio	χ^2		Number of trios
Bipolar disorder	50	32	1.56	3.95	$P = 0.047$	88
BPI	42	24	1.75	4.91	$P = 0.027$	77
BPII	8	8	1	0		11

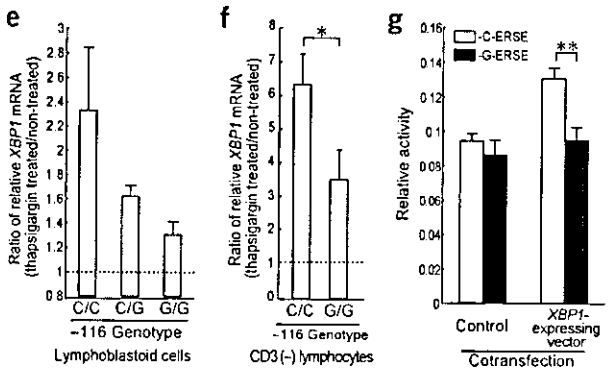


Figure 1 −116C→G polymorphism in *XBP1* is a risk factor for bipolar disorder. (**a**) −116C→G abolishes the ACGT core sequence. Numbers indicate the nucleotide positions from the transcription start site. (**b**) Association of the −116C→G polymorphism with bipolar disorder in a case-control study. (**c**) Population structure of case-control study samples. (**d**) Transmission disequilibrium test of NIMH trio samples. We used the McNemar test for statistical analysis. Estimated genotype frequency in the healthy individuals calculated from the NIMH trio samples of mixed ethnicities (mainly of European origin) was C/C, 40 (0.45); C/G, 42 (0.48); and G/G, 6 (0.07). (**e**) ER stress-induced *XBP1* mRNA expression in the cells with the genotype C/C, C/G or G/G. The ratios of relative *XBP1* mRNA levels in treated cells to that in non-treated cells are shown (values are mean ± s.e.m.). Induction levels were significantly different among genotypes ($P < 0.05$; Kruskal-Wallis test.). (**f**) ER stress-induced *XBP1* mRNA expression in the cells with the genotype C/C or G/G in non-EBV-transformed fresh CD3-negative (CD3(−)) lymphocytes (asterisk indicates $P = 0.057$ by Mann-Whitney U-test, one-tailed). (**g**) Difference in transcription activity between −116G (risk allele) and −116C (wild-type allele) in the presence of active XBP1 transcription factor. Relative activities (ratio of firefly luciferase to that of *Renilla*) are plotted (values are mean ± s.e.m.; $n = 3$). Double asterisk indicates $P < 0.05$ by two-sample *t*-test.

To test further the relevance of the ER stress response cascade to bipolar disorder, we examined the expression of *ATF6*, *XBP1* and *HSPA5* in lymphoblastoid cells by quantitative RT–PCR. In the unstressed condition, we observed no significant difference between cells from individuals with bipolar disorder and controls. After ER stress was induced by thapsigargin, however, the cells derived from individuals with bipolar disorder had a significantly smaller increase in *XBP1* and *HSPA5* mRNA levels but no difference in the *ATF6* levels (data not shown). These results suggested that the interindividual difference in the *XBP1* promoter region could be responsible for the impaired response.

Examining the upstream region, we found a putative XBP1 binding motif in addition to the ER stress-response element (Fig. 1a). XBP1 binds preferably to such a cAMP response element–like sequence, particularly containing a palindromic consensus motif ACGT (ref. 18). We sequenced the *XBP1* upstream region in samples from 648 Japanese individuals and identified the single-nucleotide polymorphism (SNP) −116C→G, changing the consensus motif ACGT into AGGT. We showed that the −116C→G SNP was significantly associated with bipolar disorder in Japanese case-control samples (Fig. 1b), in which we detected no subpopulation (Fig. 1c). The odds ratio of having the G allele for individuals with bipolar disorder was 4.6 (95% confidence interval = 2.1–10.2). The G allele was significantly over-transmitted from parents to affected offspring in the trio samples of mixed ethnicities, mainly individuals of European origin (Fig. 1d). In Japanese case-control samples, genotype frequency of individuals with bipolar disorder did not meet the Hardy-Weinberg equilibrium because the C/C genotype was rare in this group. Genotype frequency estimated by the trio samples was different from that of the Japanese individuals, and the C allele was more common than the G allele. Therefore, either the G allele is a risk allele for bipolar disorder or the C/C genotype is a protective factor.

Because the risk allele abolishes the putative XBP1 binding motif, we hypothesized that the −116C→G polymorphism might alter the positive feedback activity of XBP1. To test this hypothesis, we treated lymphoblastoid cells from healthy subjects (three with the genotype C/C, seven with C/G and five with G/G at −116 in *XBP1*) for 3 h with thapsigargin and compared the *XBP1* mRNA levels in treated and untreated cells. Induced expression of *XBP1* was higher in cells with the genotype C/C and lower in cells with G/G (Fig. 1e). We observed a similar tendency in non-transformed CD3-negative lymphocytes (Fig. 1f). Next, we measured transcriptional activity of the *XBP1* upstream region including ER stress-response element and the putative XBP1 binding site carrying either −116C or −116G. The transcriptional activity of the −116G construct was significantly lower than that of the −116C, but only when cotransfected with vector expressing active XBP1 (Fig. 1g). These findings indicated that XBP1 itself influenced the ER stress–induced expression of *XBP1* and that the −116C→G polymorphism compromises the feedback loop.

These results show that the −116C→G polymorphism of *XBP1* causes an impairment of ER stress response and increases the risk of bipolar disorder. Next, we investigated the effects of mood stabilizers on this cascade and its impairment. Mood stabilizers, such as valproate, lithium and carbamazepine, are effective in treating this illness, but the treatment response for these drugs varies among individuals[19] and the mechanism of action is debated[20]. We treated SHSY5Y neuroblastoma cells and lymphoblastoid cells with these drugs at concentrations similar to their recommended therapeutic plasma levels. Of the three mood stabilizers, only valproate had a significant effect on *ATF6* mRNA expression (Fig. 2a). To rule out the possibility that valproate caused ER stress, we examined the ratio of unspliced *XBP1* mRNA to total *XBP1* mRNA, which decreases under ER stress. But the ratio was similar before and after treatment with valproate (data not shown), indicating that valproate increases *ATF6*

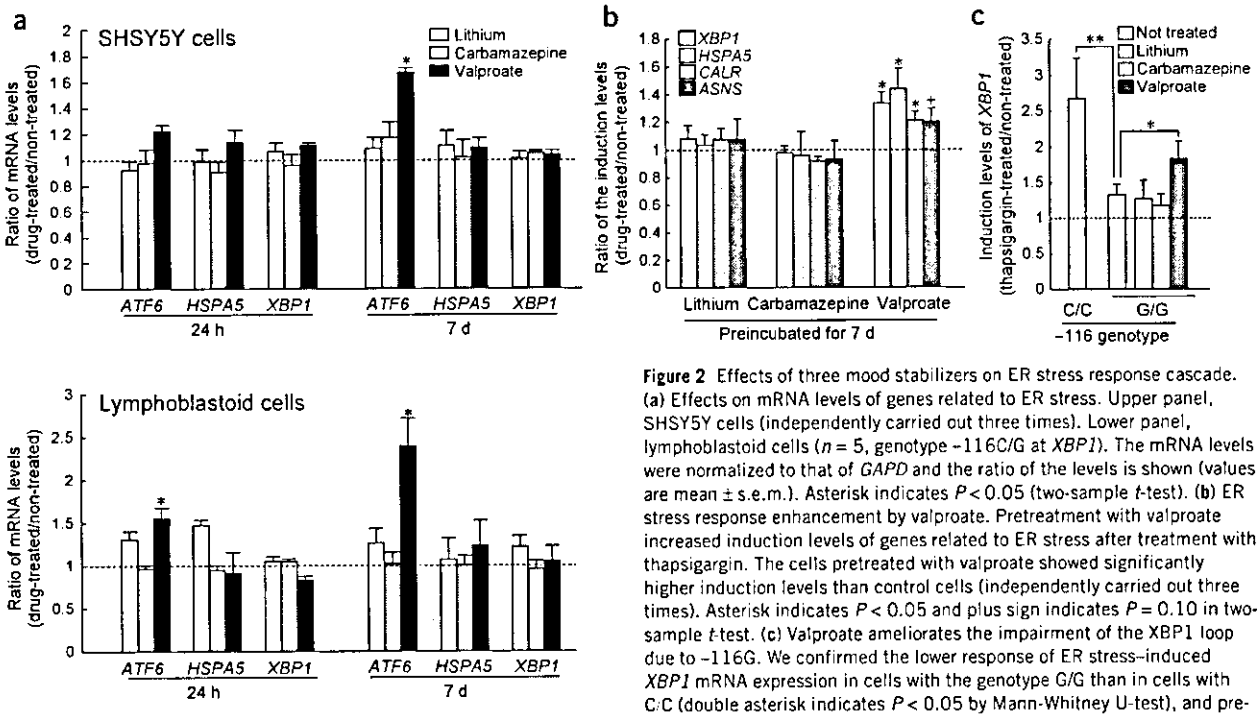


Figure 2 Effects of three mood stabilizers on ER stress response cascade. (**a**) Effects on mRNA levels of genes related to ER stress. Upper panel, SHSY5Y cells (independently carried out three times). Lower panel, lymphoblastoid cells ($n = 5$, genotype −116C/G at *XBP1*). The mRNA levels were normalized to that of *GAPD* and the ratio of the levels is shown (values are mean ± s.e.m.). Asterisk indicates $P < 0.05$ (two-sample *t*-test). (**b**) ER stress response enhancement by valproate. Pretreatment with valproate increased induction levels of genes related to ER stress after treatment with thapsigargin. The cells pretreated with valproate showed significantly higher induction levels than control cells (independently carried out three times). Asterisk indicates $P < 0.05$ and plus sign indicates $P = 0.10$ in two-sample *t*-test. (**c**) Valproate ameliorates the impairment of the XBP1 loop due to −116G. We confirmed the lower response of ER stress–induced *XBP1* mRNA expression in cells with the genotype G/G than in cells with C/C (double asterisk indicates $P < 0.05$ by Mann-Whitney U-test), and pre-incubation with valproate partially improved the induction level (asterisk indicates $P = 0.055$ by two-sample *t*-test, one-tailed).

mRNA levels but does not act as an ER stressor. Contrary to previous studies[7,8], valproate did not induce *HSPA5* mRNA levels. This may be due to the concentration of drugs used. Under therapeutic levels, valproate increased expression of *ATF6* mRNA, the upstream gene in this cascade, without affecting the expression of *HSPA5*.

Because the increase in *ATF6* mRNA should cause an increase in full-length ATF6 and a subsequent increase in cleaved ATF6 under ER stress, we hypothesized that valproate may enhance the ER stress response. To test this hypothesis, we investigated the effects of preincubation with mood stabilizers on induction of genes related to ER stress response. After culturing them for 7 d with or without mood stabilizers, we incubated the SHSY5Y cells with thapsigargin for 3 h and examined the induction of *XBP1*, *HSPA5*, *CALR* (calreticulin) and *ASNS* (asparagine synthetase) mRNA. The increased ratios (ratios of relative mRNA levels in cells treated with thapsigargin versus untreated) of the target genes were significantly higher than the controls only when preincubated with valproate (**Fig. 2b**). We further examined whether valproate could rescue the impairment of XBP1 response caused by the −116G/G genotype. As expected, the lymphoblastoid cells with the G/G genotype that were preincubated with valproate for 7 d showed significantly more induction of *XBP1* mRNA after treatment with thapsigargin for 3 h than did the control cells without valproate or the cells treated with lithium or carbamazepine (**Fig. 2c**). Although the response was still lower than that of the cells with C/C genotype, these results show that valproate ameliorates the ER stress response compromised by the risk allele −116G by reinforcing ATF6 upstream of the XBP1 loop.

Our results strongly suggest a pathophysiological role for the XBP1 loop in the ER stress response pathway in bipolar disorder. Further research on the function of XBP1 and the ER stress response pathway in the nervous system is warranted. We found that *XBP1* was relatively highly expressed in the human prefrontal cortex (roughly twice the expression found in B cells and half that in lymphoblastoid cells; data not shown). Identification of the molecular events induced by the impaired XBP1 feedback system will provide clues about the mechanisms of mood at the molecular level. Clinically, it is important that only valproate and not other mood stabilizers improved the impairment of the XBP1 loop due to −116G. Our preliminary data in a limited number of individuals with bipolar disorder suggest that the −116C→G polymorphism is associated with response to mood stabilizers (data not shown) and warrant a larger-scale clinical trial to establish customized treatment according to the genetic risk of bipolar disorder.

METHODS

Subjects. For the DNA microarray analysis, we enrolled two pairs of monozygotic twins discordant with respect to bipolar disorder (affected twins) and one pair of healthy twins. The affected twins were 49-year-old males (affected twins 1, previously reported in ref. 21) and 42-year-old males (affected twins 2). The control twins were 34-year-old males. We confirmed their monozygotic twinning using a DNA typing kit (AmpFlSTR Profiler PCR Amplification Kit, Applied Biosystems). We carried out the experiment on ER stress response on lymphoblastoid cell lines derived from five individuals with bipolar I disorder and five healthy controls. The five affected individuals were treated mainly with lithium or valproate. They were also taking various additional psychotropic agents, such as antipsychotics and antidepressants.

We examined the ER stress response difference among −116C/G genotypes on lymphoblastoid cells (three with the genotype C/C, seven with C/G and five with G/G at −116 in *XBP1*) or on lymphocytes not transformed by Epstein-Barr virus (three with the genotype C/C and four with G/G) derived from healthy subjects. The latter samples were used to confirm that the finding in lymphoblastoid cells was not confounded by the virus transformation. We divided the lymphocytes into groups of T cells and of other cells (which we considered B cells) by positive and negative selection using CD3 mineral beads (Miltenyi Biotec).

To examine whether the mood stabilizers rescued the responsiveness to ER stress in cells with the genotype −116G/G, we examined eight lymphoblastoid cells (four with the genotype C/C and four with G/G) derived from healthy subjects. The subjects for the association study of *XBP1* were 197 unrelated individuals with bipolar disorder (140 with bipolar I disorder (BPI) and 57 with bipolar II disorder (BPII)), who were tracked through at the hospitals or clinics participating in this study, and 451 unrelated control subjects who were recruited from the staffs and students of participating institutes. We made diagnosis of bipolar disorder using the DSM-IV criteria (American Psychiatric Association, 1994). For transmission disequilibrium test, we obtained 88 trio samples (77 trios with a proband with BPI and 11 trios with a proband with BPII) from National Institute of Mental Health (NIMH) genetics initiative pedigrees[22]. Written informed consent was obtained from all the subjects. The Ethics Committees of the Brain Science Institute (RIKEN) and participating institutes approved the study.

Cell cultures. We transformed lymphocytes from peripheral blood by Epstein-Barr virus and cultured them using standard techniques as described previously[23]. After transformation, we changed the culture medium every day for 3 d until use. For the DNA microarray analysis, we used cells that had not been frozen. For mRNA quantification, we extracted the RNA from cells that were frozen, thawed and recultured. We cultured SHSY5Y cells in Dulbecco's modified Eagle medium (Sigma) containing 10% fetal bovine serum. For induction of ER stress, we incubated lymphoblastoid cells with thapsigargin (300 nM). To investigate the effects of the mood stabilizers, we incubated lymphoblastoid cells or SHSY5Y cells in the medium containing lithium, valproate and carbamazepine (Wako) at their therapeutic plasma concentrations (lithium, 0.75 mM; valproate, 100 μg ml^{-1}; carbamazepine, 7.5 μg ml^{-1}) for either 24 h or 7 d. As the control experiment for carbamazepine, we added the same quantity of dimethylsulfoxide in the medium.

DNA microarray analysis. We carried out DNA microarray analysis using Hu95A Chip according to the protocols of the manufacturer (Expression Analysis Technical Manual, Affymetrix). We checked quality of total RNA or cRNA by denaturing agarose gel electrophoresis and Test2chip (Affymetrix) before experiments on Hu95A Chip. We carried out all experiments in duplicate. We normalized the expression of each gene to that of *GAPD* (glyceraldehyde 3-phosphate dehydrogenase) as a control and then compared normalized expression in each pair of twins. We analyzed only the genes assessed as 'present' using GeneSpring software (Silicon Genetics). After excluding immunoglobulin-related genes, we compared the gene expression profiles between each pair of twins and selected genes whose expression differed by a factor of 1.6 or more in both the duplicate experiments.

Quantitative PCR. We carried out real-time quantitative PCR to quantify mRNA levels and copy number of *XBP1* according to the manufacturer's protocol (Applied Biosystems). To quantify mRNA levels, we prepared single-strand cDNA by the same method used for the DNA microarray analysis. We checked the RT–PCR products by 4% agarose gel electrophoresis and confirmed that each produced a single band. We carried out an identical reaction without the reverse transcriptase to verify the absence of genomic DNA. We calculated the relative ratio by measuring ΔCt (Ct (each gene) − Ct (*GAPD*)) for each sample in quadruplicate. To quantify copy number of *XBP1*, we compared amplification of exon 4 with that of RNase P as a control (Applied Biosystems). Primer sequences are available on request.

DNA methylation. We examined the methylation pattern of *XBP1* genomic DNA in all twins using the bisulfite modification method as described[24]. We carried out bisulfite sequencing on the CpG island around exon1 of *XBP1*. We also carried out methylation-specific PCR on the CpG island before and after bisulfite modification and checked the amplification by agarose gel electrophoresis. In addition, we digested PCR products of the CpG island amplified by primers not influenced by bisulfite modification with *Bst*UI (CG/CG) and checked the methylation status by agarose gel electrophoresis. Primer sequences are available on request.

Promoter assay. We amplified a 418-bp fragment (−289 to +129) of *XBP1* by PCR and cloned it into the *Mlu*I/*Bgl*II site of pGL3-Basic vector (Promega). We prepared two kinds of reporter plasmids, carrying either −116C or −116G. We cloned the spliced *XBP1* cDNA into the *Bam*HI/*Sal*I site of pCMV-Tag3 vector (Stratagene) to construct a spliced *XBP1*-expressing vector. We transfected HeLa S3 cells cultured in a 96-well plate using Superfect (Qiagen) with 0.62 μg of DNA containing 0.3 μg of the reporter plasmid, the spliced *XBP1*-expressing vector (0 or 0.3 μg), 0.02 μg of a reference plasmid (pRL-SV40) and the pGL3-Basic vector or pCMV-Tag3 vector carrying no insert. After 36 h of incubation, we measured luciferase activities using the Dual-Glo Luciferase assay system (Promega).

Population structure. To rule out the possibility that the difference in a case-control study was influenced by hidden population stratification, we genotyped six SNPs (in *NDUFV2*, *NDUFS8*, *WFS1*, *FAAH*, *CHGB* and *FYN*), one variable-number-of-tandem-repeats site (in *C21orf2*) and one poly-A polymorphism (in *ALOX5AP*) selected from different chromosomes. We examined the population structure in 194 individuals with bipolar disorder and 240 controls using the program called structure[25]. We confirmed significant association of bipolar disorder with *XBP1* −116G, either allele-wise or genotype-wise, in these samples. We observed no subpopulation using the number of assumed subpopulation k = 2 (**Fig. 1c**) and k = 3 (data not shown).

URLs. We examined the putative XBP1 binding motif in the promoter region using the Match program (available at http://www.gene-regulation.de/) and the CpG island in the promoter region of *XBP1* using the MethPrimer program (available at http://itsa.ucsf.edu/~urolab/methprimer/).

GenBank accession numbers. Genomic sequence of *XBP1*, NT_011520.8; *NDUFV2*, NT_010859; *NDUFS8*, NT_033903; *WFS1*, NT_006051; *FAAH*, NT_004852; *CHGB*, NT_005403; *FYN*, NT_025741; *C21orf2*, NT_011515; *ALOX5AP*, NT_009799. mRNA sequence of *ATF6*, NM_007348; *XBP1*, AB076384; *XBP1* unspliced form, AB076383; *HSPA5*, X87949; *CALR*, AY047586; *ASNS*, NM_133436; *GAPD*, M33197.

Note: Supplementary information is available on the Nature Genetics website.

ACKNOWLEDGMENTS

We thank the individuals with bipolar disorder and unaffected volunteers who participated in this study. Data and biomaterials of the National Institute of Mental Health (NIMH) pedigrees were collected in four projects that participated in the NIMH Bipolar Disorder Genetics Initiative. From 1991 to 1998, the Principal Investigators and Co-Investigators were as follows: Indiana University, Indianapolis, Indiana, USA, J. Nurnberger, M. Miller and E. Bowman; Washington University, St. Louis, Missouri, USA, T. Reich, A. Goate and J. Rice; Johns Hopkins University, Baltimore, Maryland, USA, J. R. DePaulo, Jr., S. Simpson and C. Stine; NIMH Intramural Research Program, Clinical Neurogenetics Branch, Bethesda, Maryland, USA, E. Gershon, D. Kazuba and E. Maxwell.

COMPETING INTERESTS STATEMENT

The authors declare competing financial interests (see the *Nature Genetics* website for details).

Received 18 June; accepted 12 August 2003
Published online at http://www.nature.com/naturegenetics/

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Neuroscience Letters 337 (2003) 17–20

Neuroscience Letters

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Association study of the brain-derived neurotrophic factor (BDNF) gene with bipolar disorder

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Received 1 October 2002; received in revised form 21 October 2002; accepted 28 October 2002

Abstract

Brain-derived neurotrophic factor (BDNF) belongs to a family of neurotrophic factors and has been demonstrated to promote the survival, differentiation, and maintenance of a broad variety of central nervous system neurons. Several reports have suggested that the BDNF gene is a plausible functional candidate gene underlying the predisposition for developing bipolar disorder (BPD). In the present study, we investigated the possible role of the BDNF gene in the etiology of BPD using a matched case-control association design in a Japanese population. There was no evidence for an allelic or genotypic association of two polymorphisms (−1360C > T and 196G > A) of the BDNF gene with BPD. Furthermore, no significant association was observed between these polymorphisms and either of two diagnostic subtypes (bipolars I and II disorder). The results suggest that the BDNF gene is unlikely to confer susceptibility to BPD.

Keywords: Brain-derived neurotrophic factor; Bipolar disorder; Polymorphism; Association study

Bipolar disorder (BPD) is a common mental illness characterized by episodes of mania and depression. It has a lifetime prevalence of approximately 1% of the world's population. The pathophysiology and etiology of BPD remain unknown. Family, twin, and adoption studies have provided strong evidence for an important genetic component [2,13,22].

Brain-derived neurotrophic factor (BDNF) belongs to a family of neurotrophic factors that also include nerve growth factor, neurotrophin-3 and -4 [20]. It is most widely and abundantly expressed within the brain and has been demonstrated to promote the survival, differentiation, and maintenance of a broad variety of central nervous system neurons [1,8,9]. Several lines of evidence point to the BDNF gene as a reasonable candidate gene for psychiatric disorders, including BPD. Repeated administration of antidepressant drugs increases the expression of BDNF in rat brain limbic regions, particularly the hippocampus [6,15,16]. Indeed, in the human brain, Chen et al. [3] have reported that BDNF levels were increased in the hippocampus of subjects treated with antidepressants. Direct infusion of BDNF into the midbrain or the hippocampus of rats is reported to produce antidepressant effects in behavioral models of depression, including the forced swim and learned helplessness paradigms [17,18]. The results of these studies indicate that BDNF may contribute to the pathophysiology of depressive disorder, and possibly the depressive symptoms associated with BPD and schizophrenia. Furthermore, electroconvulsive treatments, which are used for the treatment of depressive disorder, BPD, and schizophrenia, also increase the expression of BDNF in the frontal cortex [5,15]. Chronic administration of lithium or valproate, which are used for the treatment of BPD, increases the expression of BDNF in the cerebral cortex

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0304-3940/02/$ - see front matter © 2002 Elsevier Science Ireland Ltd. All rights reserved.
doi:10.1016/S0304-3940(02)01292-2

Table 1
Genotype and allele frequencies of the polymorphisms of the BDNF gene[a]

−1360C > T	n	Genotype			P	Allele		P
		CC	CT	TT		C	T	
Control	190	179 (94.2)	11 (5.8)	0 (0.0)		369 (97.1)	11 (2.9)	
Total BPD	132	121 (91.7)	11 (8.3)	0 (0.0)	0.38	253 (95.8)	11 (4.2)	0.39
Bipolar I	102	94 (92.2)	8 (7.8)	0 (0.0)	0.62	196 (96.1)	8 (3.9)	0.63
Bipolar II	30	27 (90.0)	3 (10.0)	0 (0.0)	0.41	57 (95.0)	3 (5.0)	0.42
196G > A	n	Genotype			P	Allele		P
		GG	GA	AA		G	A	
Control	190	63 (33.1)	94 (49.5)	33 (17.4)		220 (57.9)	160 (42.1)	
Total BPD	130	42 (32.3)	68 (52.3)	20 (15.4)	0.85	152 (58.5)	108 (41.5)	0.94
Bipolar I	100	34 (34.0)	50 (50.0)	16 (16.0)	0.97	118 (59.0)	82 (41.0)	0.86
Bipolar II	30	8 (26.7)	18 (60.0)	4 (13.3)	0.65	34 (56.7)	26 (43.3)	0.89

[a] Numbers in parentheses indicate percentages. Statistical analysis was performed by a chi-square test or Fisher's exact test.

of the rat brain [7]. In addition, two linkage studies have suggested that chromosome 11p13–14 is a putative locus for the genes responsible for the development of BPD [4,12], and the BDNF gene is located in this region. On the basis of these evidences, we evaluated the role of the BDNF gene in BPD. In the present study, we investigated the genetic association between two different polymorphisms of the human BDNF gene and BPD through case-control studies.

One hundred and thirty-two unrelated patients with BPD (69 males and 63 females; mean age, 52.0 ± 13.9 years), including 102 with bipolar I disorder and 30 with bipolar II disorder, participated in this study. Diagnoses of BPD were made by two experienced psychiatrists, according to the Diagnostic and Statistical Manual of Mental Disorders, Fourth Edition criteria and on the basis of interviews and all available information, including hospital records. One hundred and ninety healthy volunteers (98 males and 92 females; mean age, 49.1 ± 15.1 years) were recruited as control subjects, mostly from the medical staff. Within the control group, subjects with a positive personal or familial history of major psychiatric disorders were excluded. Patients and controls were unrelated Japanese, and were individually matched for gender, age, and geographical origin. After being provided with a complete description of the study, written informed consent to participate was obtained from all participants prior to examination. This study protocol was approved by the Ethics Committee of Okayama University Graduate School of Medicine and Dentistry.

The human BDNF gene is encoded by a gene of approximately 43 kb that is located on chromosome 11p13 [11] and consists of five exons (MIM *113505). The first four exons contain putative promoter elements that control the expression of BDNF, and exon 5 contains the entire coding region for BDNF protein. In the study presented here, we investigated two kinds of single nucleotide polymorphisms (SNPs) of the human BDNF gene, −1360C > T and 196G > A. The −1360C > T polymorphism, which was detected and named C270T by Kunugi et al. [10], was localized within the 5′ untranslated region (5′UTR) of exon 1. The 196G > A polymorphism was a non-synonymous mutation that accompanied a Val66Met substitution located in exon 5. The A of the ATG-translation initiation codon is denoted nucleotide +1. The nucleotide 5′ to +1 is numbered −1. Exonic SNPs are numbered according to their positions in the coding sequence.

The genomic DNA was extracted from peripheral leukocytes by standard procedures. Polymerase chain reaction (PCR) and the PCR-based restriction fragment length polymorphism assays were performed to genotype the DNA sequence variants of the BDNF gene. PCR was carried out in a total volume of 15 μl with 10% dimethyl-sulfoxide and 0.75 units of Super*Taq* DNA polymerase (Sawady Technology Co., Japan) in the reaction mixture. The primer sequences used for analysis of −1360C > T were the forward primer 5′-CAGAGGAGCCAGCCCGGTGCG-3′ and the reverse primer 5′-CTCCTGCACCAAGCCCCATTC-3′ [10]. Those of 196G > A were 5′-ACTCTGGAGAGCGTGAATGG-3′ and 5′-ACTACTGAGCATCACCCTGGA-3′ [21]. The amplification conditions were initiated at 95°C for 5 min, followed by 35 cycles consisting of denaturation at 95°C for 1 min, annealing at the appropriate primer-pair annealing temperature for 30 s and extension at 72°C for 1 min, with a final extension step of 10 min at 72°C. The PCR products were digested at 37°C with the corresponding restriction enzyme, *Hin*fI (−1360C > T) and *Eco*72I (196G > A), and subsequently electrophoresed on 3.0% agarose gels stained with ethidium bromide. Digestion with *Hin*fI generated four fragments of 127, 63, 18, and 15 bp in subjects with −1360T allele, whereas those with −1360C allele generated three fragments of 127, 78, and 18 bp. *Eco*72I digestion produced two fragments (99 and 72 bp) in subjects with the 196G allele, whereas those with the 196A allele produced only a 171 bp fragment.

The presence of the Hardy–Weinberg equilibrium was

tested using a chi-square goodness-of-fit test. The statistical significance of differences in the genotype distribution and allele frequency between patients and controls was assessed by a chi-square test or Fisher's exact test at a significance level of 0.05, two-tailed. The level of linkage disequilibrium between two SNP sites, −1360C > T and 196G > A, was analyzed using the EH program.

Both the genotype distributions and allele frequencies for the patients and controls are shown in Table 1. The genotype distributions for patient and control groups did not deviate significantly from the Hardy–Weinberg equilibrium at these polymorphic loci. No significant differences were found in the frequency of the genotype or allele in these two polymorphisms between patients and controls (−1360C > T: genotype, $\chi^2 = 0.79$, df = 1, $P = 0.38$, allele, $\chi^2 = 0.76$, df = 1, $P = 0.39$; 196G > A: genotype, $\chi^2 = 0.32$, df = 2, $P = 0.85$, allele, $\chi^2 = 0.02$, df = 1, $P = 0.94$). With regard to the subtype of BPD, no association was observed between either of these polymorphisms and any of the diagnostic subtypes, bipolar I disorder (−1360C > T: genotype, $\chi^2 = 0.46$, df = 1, $P = 0.62$, allele, $\chi^2 = 0.44$, df = 1, $P = 0.63$; 196G > A: genotype, $\chi^2 = 0.09$, df = 2, $P = 0.97$, allele, $\chi^2 = 0.07$, df = 1, $P = 0.86$) and bipolar II disorder (−1360C > T: genotype, $\chi^2 = 0.77$, df = 1, $P = 0.41$, allele, $\chi^2 = 0.75$, df = 1, $P = 0.42$; 196G > A: genotype, $\chi^2 = 1.15$, df = 2, $P = 0.65$, allele, $\chi^2 = 0.03$, df = 1, $P = 0.89$). Pair-wise linkage disequilibrium was calculated between the two SNPs using the EH program. We found that the two SNPs were not in linkage disequilibrium with each other (Control: $\chi^2 = 5.32$, df = 3, $P = 0.15$; BPD: $\chi^2 = 5.22$, df = 3, $P = 0.16$). Accordingly, haplotype analyzes using these two SNPs were not applicable.

This study examined the possible association of two human BDNF gene polymorphisms with BPD. We genotyped the two polymorphisms of the BDNF gene, −1360C > T and 196G > A, in a Japanese population and found no association between the BDNF gene and BPD. However, our results seem to be not consistent with two recent studies reported in 2002. Neves-Pereira et al. [14] and Sklar et al. [19] showed positive association between certain haplotype of the BDNF gene and BPD by family-based association study. Their subjects were almost Caucasian, and ours were Japanese. An ethnic difference may result in these inconsistent results. As to the 196G > A polymorphism, the 196G allele frequencies in our control samples, Neves-Pereira's samples, and Sklar's samples were 0.579, 0.769, and 0.83, respectively. These data indicate that the allele frequency of the BDNF gene polymorphism is likely to differ between the two groups of different ethnicity. Therefore, the BDNF gene may confer a susceptibility to BPD in Caucasian, but not in Japanese population. However, for the following reasons we cannot definitely exclude the possibility of false negative results. First, there is a possibility of the low statistical power. In the present sample size, the statistical power to detect a small effect size (w = 0.11) was 0.80, considering an alpha value of 0.05, for detecting a significant difference in allelic distributions. As judged by the statistical power, the present total sample size was estimated to have been sufficient to reveal any statistically significant differences. However, with regard to the subtypes of BPD, especially bipolar II disorder, the power was dramatically reduced because of the limited sample size, and so our results must be qualified with a larger number of subjects. Secondly, the effect of population stratification must be taken into account. However, since all the subjects were unrelated Japanese, born and living in the middle western area of Japan, and were carefully matched for ethnicity and drawn from a population that was ethnically as homogeneous as possible, the failure to demonstrate an association was unlikely to be due to population stratification. Finally, it is possible that other as yet undetected variants of the BDNF gene may be involved in the pathogenesis of BPD. In the present study, we investigated only two polymorphisms within the 5′UTR and coding region of the BDNF gene. Therefore, it remains possible that other sequence variations in, for instance, the promoter or yet undetected 3′UTR regions, may be of importance in determining susceptibility to BPD. In addition, we found that −1360C > T and 196G > A were not in linkage disequilibrium with each other. We believe that this result is reliable because these polymorphisms were approximately 42 kb apart. However, we cannot exclude the possibility that there are other unknown polymorphisms located between −1360C > T and 196G > A, which are not in linkage disequilibrium with these two polymorphisms and confer susceptibility to BPD.

In conclusion, the results of this study do not support a possible association of the BDNF gene with susceptibility to BPD. Further studies are required to clarify whether any as yet unidentified functional mutation in the BDNF gene is involved in the etiology of BPD.

The present study was supported in part by a grant from Zikei Institute of Psychiatry (Okayama, Japan).

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ORIGINAL ARTICLES

Association Between Chromogranin B Gene Polymorphisms and Schizophrenia in the Japanese Population

Yoshimi Iijima, Toshiya Inada, Tsuyuka Ohtsuki, Hisashi Senoo, Masaki Nakatani, and Tadao Arinami

Background: *We found in previous work a significant association between schizophrenia and D20S95 on chromosome 20p12.3. In this study, we analyzed 10 microsatellite markers and found an association of schizophrenia with D20S882 and D20S905 that flank D20S95. The chromogranin B gene* (CHGB) *is 30 kb from D20S905. The chromogranin B (secretogranin I) belongs to a series of acidic secretory proteins that are widely expressed in endocrine and neuronal cells, and its cerebrospinal fluid levels have been reported to decrease in patients with chronic schizophrenia.*

Methods: *We screened for polymorphisms in* CHGB *with polymerase chain reaction direct sequencing methods in 24 Japanese schizophrenic patients and identified a total of 22 polymorphisms. Allelic and genotypic distributions of detected polymorphisms were compared between unrelated Japanese schizophrenic patients* (n = *192) and healthy control subjects* (n = *192).*

Results: *Statistically significant differences in the allelic distributions were found between schizophrenic patients and control subjects for 1058C/G (A353G) (corrected* p = 7.7×10^{-5}*) and 1104A/G (E368E) (corrected* p = 8.1×10^{-6}*). The 1058C/G and 1104A/G alleles were in almost complete linkage disequilibrium and were in linkage disequilibrium with D20S95.*

Conclusions: *Results suggest that the* CHGB *variations are involved in the susceptibility to schizophrenia in our study population.*

Key Words: Schizophrenia, chromogranin B, association study, neuropeptide

The region of 20p12.3-p11 may contain a locus of predisposition to schizophrenia (Lewis et al 2003). In our previous screening association study, we observed a significant association with schizophrenia at the locus of D20S95 ($p = 5 \times 10^{-6}$, corrected p value after Bonferroni correction, .00035) on 20p12.3 (Kitao et al 2000). The marker D20S95 is approximately 2 megabases (Mb) outside the 21.2 to 47.5 cM region of chromosome 20 highlighted in the meta-analysis of Lewis et al (2003), though quite possibly within its confidence bounds. In the individual genome scans, Moises et al (1995) reported a p-value of .009 with marker D20S40 and Hovatta et al (1998) found a maximum lod score of 1.22 with marker D20S172. These markers are located approximately 7 Mb and 12 Mb centromeric to D20S95.

The only known gene within 180 kb from D20S95 is the gene encoding chromogranin B, a tyrosine-sulfated secretory protein found in a wide variety of peptidergic endocrine cells. The granins (secretogranins/chromogranins) belong to a family of soluble proteins stored and released from the large dense-core secretory vesicles of the synapse (Benedum et al 1987; Winkler and Fischer-Colbrie 1992). A number of studies have compared the chromogranin levels in cerebrospinal fluid (CSF) between healthy control subjects and schizophrenic patients at various stages of this disease (Landen et al 1999; Miller et al 1996; van Kammen et al 1991, 1992, 1994). Landen et al (1999) reported that levels of chromogranin A and chromogranin B were lower in chronic schizophrenic patients and pointed out the possibility that an acute increase of chromogranin levels reflects an active disease process and a chronic decline indicates an advanced neurodegenerative process. Reduction of chromogranin B-like immunoreactivity in distinct subregions of the hippocampus from individuals with schizophrenia was reported (Nowakowski et al 2002). An association between some *CHGB* polymorphisms and schizophrenia was reported in the Chinese Han population (Zhang et al 2002).

In the present study, we performed additional dense-mapping analyses using 10 microsatellite markers close to D20S95 and the mutation search of the *CHGB*, followed by the case-control association studies on its detected polymorphisms in schizophrenic patients, using the same sample set as we previously used in the screening study in which a significant association with schizophrenia was detected at D20S95.

Methods and Materials

Ethical Considerations

The present study was approved by the Ethical Committee of the Kohnodai area, National Center of Neurology and Psychiatry, University of Tsukuba, and Nagoya University. Written informed consent was obtained from all subjects.

Subjects

The DNA samples were all selected from those of the subjects enrolled in our previous screening study (Kitao et al 2000). The subjects consisted of 192 schizophrenic patients and 192 healthy control subjects. The schizophrenic patients (91 men, 101 women; aged 19–90 years [mean 57.2 years]) were all inpatients recruited from several psychiatric facilities located around the Tokyo area. They were interviewed several times during a hospitalization period of 6 months or more by experienced psychiatrists who were familiar with the structured clinical interview for DSM-III-R (SCID) rating system (Spitzer et al 1992). The diagnosis of schizophrenia was assigned on the basis of clinical interviews and chart review of medical records, accord-

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Received October 27, 2003; revised March 8, 2004; accepted March 10, 2004.

0006-3223/04/$30.00
doi:10.1016/j.biopsych.2004.03.012

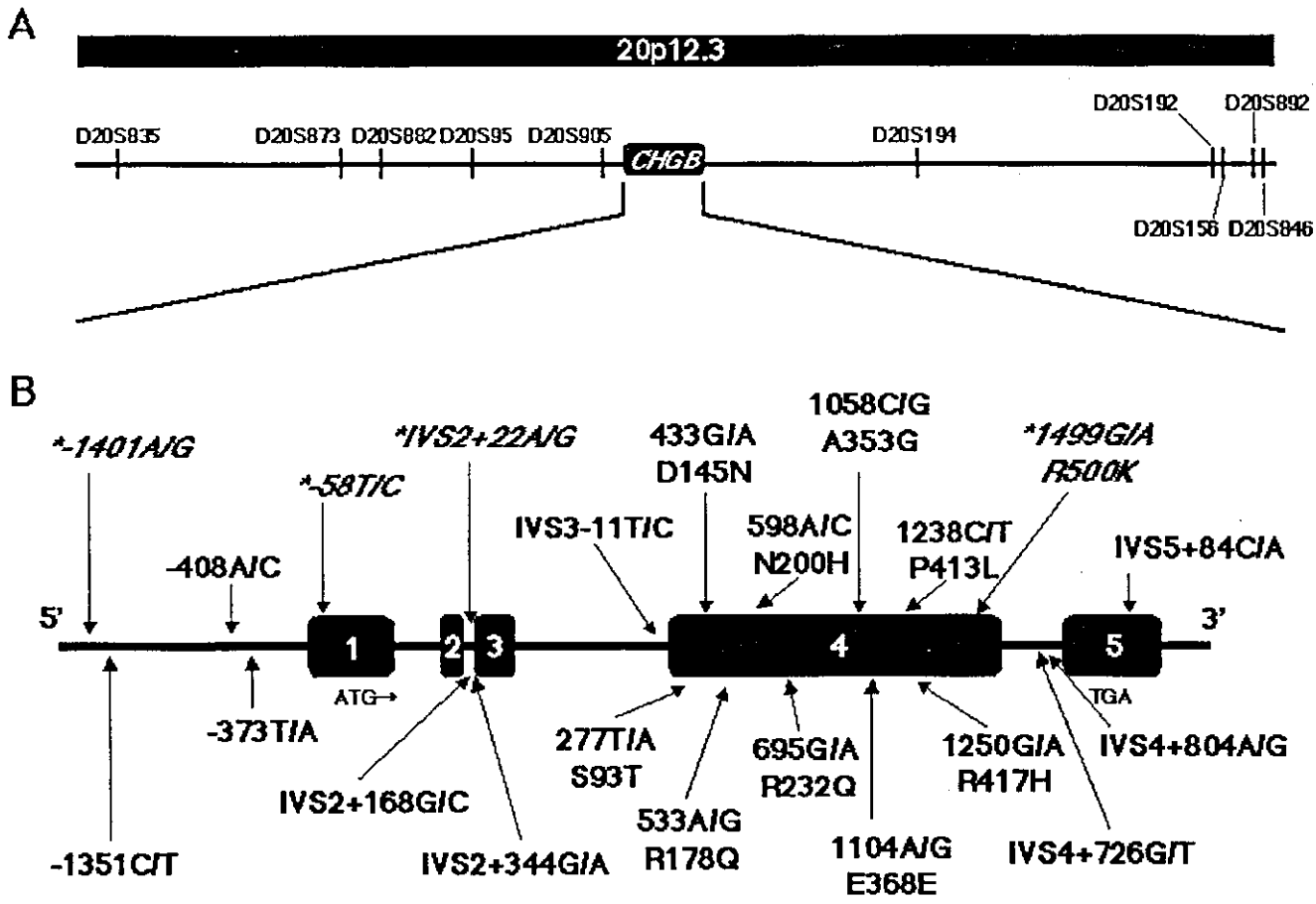

Figure 1. **(A)** The positions of the microsatellite markers in relation to chromogranin B gene (*CHGB*). **(B)** Genomic organization and positions of the SNPs in *CHGB*. *Italics are newly identified SNPs. SNP, single nucleotide polymorphism.

Table 1. Results of the Additional Dense-Mapping Analyses Using 10 Microsatellite Markers Close to D20S95

Microsatellite Markers[a]	Distance from *CHGB* (kb)	Number of Subjects		Statistics[b]	
		Schizophrenia	Control	H-W[c]	S vs. C[d]
D20S835	−577	172	192	ns	ns
D20S873	−295	175	191	ns	ns
D20S882	−257	203	196	.02	.02
D20S95	−176	230	217	ns	.002
D20S905	−29	184	190	ns	.04
CHGB	0				
D20S194	251	164	135	ns	ns
D20S192	805	171	192	ns	ns
D20S156	809	171	192	ns	ns
D20S892	858	174	192	ns	ns
D20S846	873	176	192	ns	ns

Details are shown in Appendix 1.

CHGB, chromogranin B gene; kb, kilobase; H-W, Hardy-Weinberg equilibrium; S, schizophrenia; C, control.

[a]These markers are listed in the order from telomeric (D20S835) to centromeric (D20S846) site. The average distance between the markers is about 120 kb.

[b]*p* value is denoted when $p < .05$.

[c]Deviations from the Hardy-Weinberg equilibrium.

[d]Comparison between schizophrenia vs. control (empirical *p*).

ing to the DSM-III-R criteria (American Psychiatric Association 1987). We actually did not use SCID itself at the clinical interview because: (1) a validated Japanese translation version of SCID was not available at the time of clinical evaluation in this study, and (2) we considered that diagnosis by repeated interviews by trained attendant physicians seems to be more reliable than SCID performed by a nonattendant physician only at once. Control subjects (96 men, 96 women; aged 24–87 years [mean 49.8 years]) were recruited mostly from the medical staff working in the psychiatric facilities and had no history of psychoses. All subjects were of Japanese descent, born to Japanese parents. The sampling methods of the subjects were satisfied with the criteria of the proposed checklists for gene-disease association study (Colhoun et al 2003; Little et al 2002).

Genomic Procedure

Genomic DNA was extracted from leukocytes in venous blood samples. Ten microsatellite markers on chromosome 20p12.3, D20S835 (Genome Database [GDB]:603368), D20S873 (GDB:609813), D20S882 (GDB:610170), D20S905 (GDB:612744), D20S916 (GDB:614700), D20S194 (GDB:200327), D20S192 (GDB:200199), D20S156 (GDB:198517), D20S892 (GDB:611556),

Table 2. Pair-Wise Linkage Disequilibrium in the *CHGB*

	4 −1401A/G	5 −1351C/T	6 −408A/C	7 −373T/A	8 −58T/C	9 IVS3-11T/C	10 277T/A	11 433G/A	12 533A/G	13 598A/C	14 695G/A	15 1058C/G	16 1104A/G	17 1238C/T	18 1250G/A	19 1499G/A
4	−1401A/G		1.00	1.00	.73	.51	.60	1.00	1.00	1.00	1.00	.50	.48	.37	.69	.84	.60
5	−1351C/T	.10		.90	.72	.43	1.00	1.00	.98	.85	.92	.64	.64	.57	.58	.93
6	−408A/C	.01	.35		1.00	.96	.77	.86	.45	.88	1.00	.67	.84	.84	.77	.83	.90
7	−373T/A	.00	.09	.27		.58	1.00	.72	.85	.76	.53	.51	.68	.70	.55	1.00	1.00
8	−58T/C	.00	.02	.45	.01		.50	.36	.44	.42	.74	.43	.64	.63	1.00	.66	1.00
9	IVS3-11T/C	.00	.07	.07	.02	.00		1.00	1.00	1.00	1.00	.98	.61	.62	.83	.74	.51
10	277T/A	.01	.70	.23	.07	.01	.06		.99	.99	1.00	.90	.58	.57	.56	.51	.92
11	433G/A	.01	.38	.20	.08	.08	.17	.37		1.00	1.00	.92	.47	.46	.23	.43	.67
12	533A/G	.02	.58	.49	.14	.04	.11	.60	.60		1.00	.94	.63	.62	.32	.42	.83
13	598A/C	.13	.06	.10	.02	.04	.01	.08	.07	.12		1.00	.79	.79	.54	.43	.90
14	695G/A	.00	.06	.06	.01	.02	.29	.06	.16	.09	.01		.58	.59	.74	.69	.48
15	1058C/G	.00	.13	.57	.16	.29	.05	.08	.15	.21	.04	.02		1.00	1.00	.98	.77
16	1104A/G	.00	.13	.57	.16	.29	.05	.08	.15	.21	.04	.04	1.00		1.00	.98	.77
17	1238C/T	.02	.04	.08	.02	.04	.00	.04	.00	.01	.29	.00	.10	.10		.75	.81
18	1250G/A	.01	.25	.34	.11	.19	.04	.16	.12	.17	.02	.03	.66	.66	.03		.82
19	1499G/A	.01	.05	.05	.02	.03	.31	.04	.09	.07	.01	.24	.10	.10	.01	.04	

Upper diagonal figures are D' and lower diagonal figures are r^2.
CHGB, chromogranin B gene.

and D20S846 (GDB:606163), were amplified by polymerase chain reaction (PCR). The positions of these markers in relation to *CHGB* is shown in Figure 1A. The PCR primers are available on the GDB (http://gdbwww.gdb.org). Genotyping to determine the lengths of the microsatellite alleles was done with an ABI PRISM 3100 DNA sequencer (Applied Biosystems, Foster City, California). The sequence and genomic structure of *CHGB* were obtained from the high throughput genome sequences (HTGs) database of the Blast server at the National Center for Biotechnology Information (NCBI) (http://www.ncbi.nlm.nih.gov/BLAST/). All exons containing exon-intron junctions and a 5′-side regulatory region of *CHGB* were amplified by the PCR method. The primers are available on request. Polymorphisms were screened in 24 schizophrenic patients by direct sequencing of PCR products with the Big Dye Terminator Cycle Sequencing kit (Applied Biosystems, Foster City, California) and on the ABI PRISM 3100 DNA sequencer. All nucleotide variants detected in this study were genotyped by direct sequencing after PCR amplification. Trace data were aligned by Sequencher software (Gene Codes Corp, Ann Arbor, Michigan) and scanned for polymorphisms.

Statistical Procedures

Microsatellite Marker Analyses. Assessing the deviations from Hardy-Weinberg equilibrium and the case-control comparison of the distribution of the microsatellite allele frequencies between control subjects and schizophrenic patients were performed by the program implemented in MEGA2 (Guo and Thompson 1992).

Single Nucleotide Polymorphism Analyses. Deviations of the genotype distributions from the Hardy-Weinberg equilibrium were assessed with the 2×3 χ^2 test. Case-control comparisons of genotype and allele frequencies of polymorphisms were done with the Armitage Trend Test and 2×2 χ^2 tests. We examined associations by permutation procedure in COCAPHASE ver 2.4 to determine the empirical significance of our findings. We did not use -EM and -missing options. The χ^2 value, the p-value, and the odds ratio (OR) were calculated with the SPSS computer package for Windows Release 11.0J (SPSS Japan Inc., Tokyo, Japan). Linkage disequilibrium analyses were conducted with the Cocaphase program: unbiased application of the transmission/disequilibrium test to multilocus haplotypes (Dudbridge et al 2000).

Results

Results of the additional dense mapping association analyses using 10 microsatellite markers close to D20S95 on 20p12.3 with schizophrenia are shown in Table 1 and supplemental data table 1. The genotype frequencies of these microsatellite markers did not significantly deviate from Hardy-Weinberg equilibrium except for D20S882 ($P = 0.02$). As shown in Table 1, possible association with schizophrenia was detected in the loci of D20S882 and D20S905, in addition to D20S95. The 109-bp allele of D20S95 was more frequent in the patient group (.10) than in the control group (.03, uncorrected $p = 7.2 \times 10^{-5}$, empirical $p = .002$). The 80-bp allele of D20S882 was less frequent in the patient group (.14) than in the control group (.22, uncorrected $p = .0009$, empirical $p = .02$). The 90-bp allele of D20S905 was less frequent in the patient group (.01) than in the control group (.05, uncorrected $p = .002$, empirical $p = .004$). Although there was a global significance for the haplotype comprising the three microsatellites ($p = 1 \times 10^{-9}$), no specific individual haplotype of these microsatellite markers was significantly associated with schizophrenia after correction for the number of haplotypes.

Table 3. Genotype and Allelic Distribution of the *CHGB* SNPs in Japanese Control Subjects and Patients with Schizophrenia

		Genotype					Allele			
					p Value[a]					
Polymorphisms Group	*N*	Genotype Count			H-W[c]	S vs. C[d]	Count (frequency)		Odds Ratio (95% CI)	*p* Value[b] S vs. C[d]
−1401A/G		AA	AG	GG			A	G		
Control	192	186	6	0	1.00	.79	378 (.98)	6 (.02)	1.22 (.40–3.65)	.73
Schizophrenia	185	178	7	0	1.00		363 (.98)	7 (.02)		
−1351C/T		CC	CT	TT			C	T		
Control	192	78	77	37	.30	.87	233 (.61)	151 (.39)	.92 (.69–1.23)	.58
Schizophrenia	186	72	89	25	.98		233 (.63)	139 (.37)		
−408A/C		AA	AC	CC			A	C		
Control	96	14	45	37	1.00	.47	73 (.38)	119 (.62)	.79 (.53–1.19)	.26
Schizophrenia	95	15	53	27	.67		83 (.44)	107 (.56)		
−373T/A		TT	TA	AA			T	A		
Control	94	71	21	2	.99	.43	163 (.87)	25 (.13)	.99 (.55–1.79)	.97
Schizophrenia	95	70	25	0	.33		165 (.87)	25 (.13)		
−58T/C		TT	TC	CC			T	C		
Control	185	106	67	12	.96	.24	279 (.75)	91 (.25)	1.22 (.88–1.69)	.24
Schizophrenia	181	94	71	16	.95		259 (.72)	103 (.28)		
IVS3-11T/C		TT	TC	CC			T	C		
Control	187	154	32	1	.84	.32	340 (.91)	34 (.09)	.66 (.38–1.14)	.14
Schizophrenia	186	164	21	1	.99		349 (.94)	23 (.06)		
277T/A (S93T)		TT	TA	AA			T	A		
Control	189	73	86	30	.91	.82	232 (.61)	146 (.39)	.94 (.70–1.26)	.68
Schizophrenia	187	73	89	25	.98		235 (.63)	139 (.37)		
433G/A (D145N)		GG	GA	AA			G	A		
Control	187	73	90	24	.96	.78	236 (.63)	138 (.37)	.99 (.73–1.33)	.94
Schizophrenia	187	77	83	27	.91		237 (.63)	137 (.37)		
533A/G (R178Q)		AA	AG	GG			A	G		
Control	188	51	90	47	.92	.35	192 (.51)	184 (.49)	.87 (.65–1.16)	.34
Schizophrenia	187	42	94	51	1.00		178 (.48)	196 (.52)		
598A/C (N200H)		AA	AC	CC			A	C		
Control	189	149	39	1	.82	.59	337 (.89)	41 (.11)	1.12 (.72–1.76)	.61
Schizophrenia	187	143	43	1	.57		329 (.88)	45 (.12)		
695G/A (R232Q)		GG	GA	AA			G	A		
Control	186	157	28	1	1.00	.45	342 (.92)	30 (.08)	.87 (.50–1.50)	.61
Schizophrenia	184	161	20	3	.50		342 (.93)	26 (.07)		
1058C/G (A353G)		CC	CG	GG			C	G		
Control	192	49	89	54	.80	1.3×10^{-5}	187 (.49)	197 (.51)	1.96 (1.46–2.63)	5.9×10^{-6}
Schizophrenia	190	21	82	87	.97		124 (.33)	256 (.67)		
1104A/G (E368E)		AA	AG	GG			A	G		
Control	192	49	89	54	.80	2.0×10^{-6}	187 (.49)	197 (.51)	2.11 (1.57–2.84)	6.2×10^{-7}
Schizophrenia	187	20	76	91	.89		116 (.31)	258 (.69)		
1238C/T (P413L)		CC	CT	TT			C	T		
Control	192	155	36	1	.82	.54	346 (.90)	38 (.10)	1.15 (.72–1.83)	.55
Schizophrenia	187	147	38	2	1.00		332 (.89)	42 (.11)		
1250G/A (R417H)		GG	GA	AA			G	A		
Control	192	71	84	37	.67	.006	226 (.59)	158 (.41)	1.51 (1.13–2.01)	.005
Schizophrenia	187	42	98	47	.90		182 (.49)	192 (.51)		
1499G/A (R500K)		GG	GA	AA			G	A		
Control	191	160	29	2	.84	.270	349 (.91)	33 (.09)	.67 (.38–1.16)	.15
Schizophrenia	186	166	18	2	.75		350 (.94)	22 (.06)		

CHGB, chromogranin B gene; H-W, Hardy-Weinberg equilibrium; S, schizophrenia; C, control; CI, confidence interval.
[a]Armitage's trend test.
[b]Chi-squared test.
[c]H-W: Observed genotype vs. expected genotype according to Hardy-Weinberg equilibrium.
[d]S vs. C: Genotype comparison between schizophrenia vs. control.

All exons containing exon-intron junctions and a 5′-side regulatory region of *CHGB* were screened for polymorphisms in 24 schizophrenic patients. A total of 22 single nucleotide polymorphisms (SNPs) were identified (Figure 1B). Eighteen SNPs, −1351C/T (rs236139), −408A/C (rs236140), −373T/A (rs236141), IVS2+168G/C (rs236145), IVS2+344G/A (rs236146), IVS3−11T/C (rs6139872), 277T/A(S93T) (rs6085324), 433G/A(D145N) (rs6133278), 533G/A(R178Q) (rs910122), 598A/C(N200H) (rs881118), 695G/A(R232Q) (rs6139873), 1058G/C(A353G) (rs236152), 1104G/A(E368E) (rs236153), 1238C/

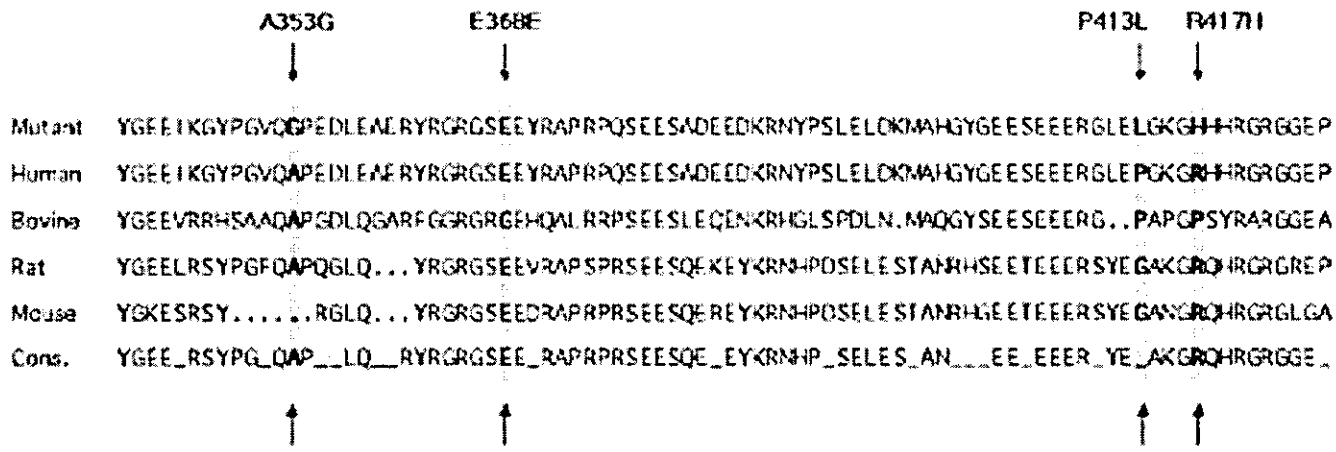

Figure 2. Comparison of the amino acid sequences of chromogranin B from human, bovine, rat, and mouse. Cons. is calculated consensus sequence. Dots (.) denote deletions in the sequence.

T(P413L) (rs742710), 1250G/A(R417H) (rs742711), IVS4+726G/T (rs236154), IVS4+804G/A (rs236155), and IVS5+84C/A (rs2821), are listed in the database for single nucleotide polymorphisms (dbSNP) in the NCBI (http://www.ncbi.nlm.nih.gov/SNP/). Three SNPs, −1401A/G, IVS2+22A/G, and 1499G/A(R500K), not listed in the dbSNP database were identified in the mutation search of *CHGB* in the Chinese Han population (Zhang et al 2002). Seven SNPs, −1095G/C (rs989462), −1042G/A (rs6085321), 402G/C(K177N) (rs236150), 727G/A(T423A) (rs236151), 1466C/T(L489L) (rs6107717), IVS4+9T/C (rs617000), and IVS5+277A/T (rs 236156), which are listed in the dbSNP database, were not detected in the Japanese population. One SNP, −58T/C, was newly identified in this study. Among these SNPs, 16 at the coding or regulatory regions were selected for further association study.

Linkage disequilibrium was found in many pairs of the 16 SNPs (Table 2). The genotype and allele frequencies of all SNPs are shown in Table 3. The genotype distributions did not deviate significantly from those expected according to the Hardy-Weinberg equilibrium for polymorphisms. Statistically significant differences in allele distributions were found between schizophrenic patients and healthy control subjects for 1058C/G ($p = 5.9 \times 10^{-6}$, empirical $p = 8.2 \times 10^{-5}$, OR = .510, 95% confidence interval [CI] = .38–.68) and 1104A/G ($p = 6.2 \times 10^{-7}$, empirical $p = 1 \times 10^{-5}$, OR = .474, 95% CI = .35–.64), and a trend of association was found between 1250G/A and schizophrenia ($p = .005$, empirical $p = .05$, OR = 1.51, 95% CI = 1.13–2.01) (Table 2). The genotype distributions of these three SNPs suggest the presence of a gene dose-response effect: a linear trend in disease risk with number of copies of the high-risk allele (uncorrected $p = 1.3 \times 10^{-5}$, uncorrected $p = 2.0 \times 10^{-6}$, and uncorrected $p = .006$, respectively). The frequency of the risk GG genotype of A353G in the control subjects was .28 and its OR for schizophrenia was 2.1 (95% CI = 1.4–3.3) compared with other genotypes. Two SNPs, 1058C/G and 1104A/G, were in almost complete linkage disequilibrium (Table 2), and almost the same p-value was obtained for the haplotype associations with schizophrenia (uncorrected $p = 6.1 \times 10^{-7}$). Other haplotypes are also associated with schizophrenia; however, they are mainly due to 1058C/G and 1104A/G polymorphisms (Table 4). Linkage disequilibrium was significant ($p < .05$) between SNPs associated with schizophrenia, namely 1058C/G (D′ = .43), 1104A/G (D′ = .44), and 1250G/A (D′ = .37) in the *CHGB* and the 109-bp allele of D20S95.

Although it has been reported that the significant association with schizophrenia ($p < .001$) was observed at two SNPs of 433G/A and 533A/G in the Chinese Han population, these findings were not replicated in our present study. The power of this study to replicate the findings from the Chinese-Han population was 90%.

Discussion

Our hypothesis that the chromogranin B gene (*CHGB*) is a plausible positional candidate for association with schizophrenia can be strongly supported by the results of our additional dense mapping analyses examined in the present study. This hypothesis is further supported by the findings in our subsequent association study between *CHGB* and schizophrenia: a significant association of 1058C/G and 1104A/G, and a trend of association of 1250G/A. These SNPs were in linkage disequilibrium with the 109-bp allele of D20S95. Association study may lead to false-positive and false-negative signals because of genetic stratification or population subdivision. Our subjects were all of Japanese descent living in the same area and we have no evidence of population stratification in our case or control samples. As shown in a previous report, Japanese is a relatively homogeneous population with no stratification (Daimon et al 2003; Kakiuchi et al 2003). However, unknown population stratification would not be excluded.

There is another Chinese study showing a significant association between some SNPs of *CHGB* and schizophrenia. Although our study and a study of Zang et al (2002) found significant associations between *CHGB* SNPs and schizophrenia, the associated SNPs differed between the two studies. Significant associations with schizophrenia were found at two SNPs, 433G/A ($p = .004$, the G allele was associated with schizophrenia, OR = 1.60, 95% CI = 1.17–2.18) and 533A/G ($p = .0005$, the A allele was associated with schizophrenia, OR = 1.73, 95% CI = 1.28–2.18), in the Chinese Han population (Zhang et al 2002). We failed to find these associations in the present study, 433G/A (OR = 1.01, 95% CI = .75–1.36) and 533A/G (OR = .87, 95% CI = .65–1.16). The ORs between the two studies significantly differed ($p = .04$ for 433G/A and $p = .001$ for 533G/A, respectively). The alleles

Table 4. Haplotypes Showing Most Significant Association with Schizophrenia per Each Window

Haplotype Window	433 G/A	533A/G	598A/C	695G/A	1058C/G	1104A/G	1238C/T	1250G/A	1499G/A	Haplotype Frequency (%) Schiz	Control	*p* Values Permutation Global	Permutation Global	Permutation Individual
Window 2					C	A				.31	.49	6.6×10^{-6}	.00009	6.6×10^{-6}
Window 3					C	A	C			.31	.49	5.9×10^{-5}	.0007	1.0×10^{-5}
Window 4				G	C	A	C			.27	.43	.0007	.01	2.0×10^{-5}
Window 5			A	G	C	A	C			.27	.40	.003	.04	.0001
Window 6		A	A	G	C	A	C			.03	.12	.005	.04	.002

Schiz, schizophrenia

that found to be associated with schizophrenia in the Chinese population, the G allele of the 433G/A polymorphism and the A allele of the 533A/G polymorphism, were on the haplotypes negatively associated with schizophrenia in our Japanese population. Linkage disequilibrium between 1058C/G and 433G/A or 533A/G were not observed (D′ = .47, D′ = .63, respectively). Our Japanese study tested more SNPs, and the most interesting SNPs were not tested in the larger Chinese sample. The SNPs significantly associated with schizophrenia in the Chinese population, however, also were nonsynonymous and of potential interest. Although it is possible that the apparent discrepancy between our study and that of Zhang et al (2002) occurred by chance, it is also possible that the discrepancy is due to ethnic differences or differences in the clinical characteristics of schizophrenic patients. We have observed that *CHGB* SNPs are associated with extrapyramidal side effects and clinical symptoms (unpublished data). Therefore, further studies are necessary to address the discrepancy between the Japanese and Chinese studies. Associations between *CHGB* and schizophrenia have been found in two independent studies of East Asian populations, indicating that *CHGB* is a plausible association with susceptibility to schizophrenia and is one of the genes contributing to the linkage of 20p12.3-p11 to schizophrenia found by meta-analysis (Lewis et al 2003).

The granin family plays an important role in the sorting and aggregation of secretory products in the trans-Golgi network (Ozawa and Takata 1995). Chromogranin A, chromogranin B (secretogranin I), and chromogranin C (secretogranin II) are well known. Chromogranin B also localizes to the nucleus and controls transcription of many genes, including those for transcription factors (Yoo et al 2002). Recently, chromogranin B was reported to have an important role in the intracellular calcium signaling in neurons by interacting with IP3 receptor in a pH-dependent manner (Thrower et al 2003). Human chromogranin B consists of 657 amino acids (Benedum et al 1987). Since the function of chromogranin B is still a matter of debate, it is acceptable that no functional analysis was performed for the 1058G/C(A353G). However, the amino acid 353A and 417R are relatively conserved among species, suggesting that the substitution has some impact on the protein function (Bauer and Fischer-Colbrie 1991) (Figure 2).

Because two of three SNPs we found to be associated with schizophrenia were nonsynonymous, they might be related directly to the susceptibility to schizophrenia. It will be important to replicate the finding in independent samples of schizophrenia of the Japanese population. However, it is possible that these SNPs are just in linkage disequilibrium with other polymorphisms responsible for giving the disease susceptibility. Our findings represent an initial step toward an understanding of the possible etiologic role that *CHGB* plays in schizophrenia. It will be interesting to consider the functional changes in the chromogranin B proteins derived from its genetic variations that may have roles in the clinical phenotypes of schizophrenia. Further research is needed with transgenic mice to clarify the exact role of chromogranin B in the pathophysiology of schizophrenia, especially to identify the functional changes derived from the genetic variations of *CHGB*. Clinical studies are also required to evaluate the psychopathological features in schizophrenic patients who have biochemical changes in chromogranin B levels and *CHGB* variations.

This study was supported in part by the research grants (8A-1 and 9B-5) for nervous and mental disorders from the Japanese Ministry of Health, Labor and Welfare and by the grants-in-aid for scientific research (No. 07770817, 08770805, 09770770, 1167094, and 15591217) from the Japanese Ministry of Education and Science. A fellowship to YI from the Japan Society for the Promotion of Science for Young Scientists is also acknowledged.

A part of the present study was presented at the 12th World Congress of Psychiatry, August 24–29, 2002, Yokohama, Japan.

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Appendix 1. Supplemental Data for Table 1. Case-Control Association Analyses with Microsatellite Markers on Chromosome 20p

	Individual Allele					Global		
Marker	Allele (bp)	Case	Control	Chi-square	*p*	LRS	DF	*p*
D20S835						8.5	10	.58
	195	.169	.193	.712	.399			
	197	.003	.000	1.501	.221			
	199	.009	.013	.313	.576			
	201	.015	.013	.031	.861			
	203	.142	.138	.029	.864			
	205	.349	.388	1.197	.274			
	207	.087	.073	.505	.478			
	209	.017	.026	.633	.426			
	211	.119	.096	.987	.321			
	213	.087	.060	2.004	.157			
	215	.003	.000	1.501	.221			
D20S873						7.9	7	.34
	185	.000	.008	3.913	.048			
	187	.291	.270	.430	.512			
	189	.003	.010	1.685	.194			
	191	.100	.105	.044	.834			
	193	.026	.018	.466	.495			
	195	.537	.545	.040	.842			
	197	.034	.042	.288	.592			
	199	.009	.003	1.236	.266			
D20S882						21.3	7	.003
	72	.037	.031	.245	.620			
	74	.335	.247	7.420	.006			
	76	.126	.181	4.759	.029			
	78	.355	.316	1.316	.251			
	80	.136	.219	9.711	.002			
	82	.007	.005	.169	.681			
	84	.002	.000	1.353	.245			
	86	.002	.000	1.353	.245			
D20S95						116.6	11	.000
	95	.000	.002	1.148	.284			
	97	.009	.103	48.970	.000			
	99	.124	.111	.411	.522			
	101	.093	.022	26.940	.000			
	103	.052	.032	2.672	.102			
	105	.100	.192	17.630	.000			
	107	.339	.320	.436	.509			
	109	.098	.027	24.160	.000			
	111	.141	.148	.098	.754			
	113	.028	.020	.720	.396			
	115	.011	.020	1.480	.224			
	117	.004	.003	.065	.798			
D20S905						24.9	11	.009
	76	.003	.003	.001	.982			
	78	.038	.042	.080	.777			
	80	.008	.016	.937	.333			
	82	.014	.016	.063	.802			
	84	.264	.311	2.014	.156			
	86	.429	.376	2.187	.139			
	88	.223	.174	2.848	.092			
	90	.014	.053	9.457	.002			
	92	.000	.005	2.714	.099			
	94	.008	.000	4.268	.039			
	96	.000	.003	1.356	.244			
	98	.000	.003	1.356	.244			
D20S194						17.7	21	.67
	185	.003	.000	1.442	.230			
	187	.011	.003	2.105	.147			
	189	.011	.013	.065	.799			
	191	.003	.005	.290	.591			
	193	.025	.044	2.167	.141			
	195	.060	.065	.069	.793			
	197	.047	.047	.000	.991			
	199	.069	.063	.117	.733			
	201	.052	.076	1.707	.191			
	203	.088	.073	.569	.451			
	205	.146	.130	.373	.541			
	207	.107	.138	1.659	.198			
	209	.146	.117	1.325	.250			
	211	.060	.060	.001	.975			
	213	.096	.081	.553	.457			
	215	.025	.034	.549	.459			
	217	.016	.026	.826	.364			
	219	.019	.013	.458	.499			
	221	.008	.003	1.162	.281			
	223	.003	.003	.001	.970			
	225	.000	.005	2.672	.102			
	227	.005	.003	.397	.529			
D20S192						12.1	12	.44
	273	.000	.003	1.275	.259			
	275	.003	.000	1.507	.220			
	277	.012	.005	.939	.333			
	281	.015	.010	.261	.610			
	283	.003	.003	.007	.935			
	285	.436	.471	.929	.335			
	287	.020	.031	.838	.360			
	289	.088	.081	.115	.735			
	291	.114	.122	.121	.728			
	293	.298	.255	1.677	.195			
	295	.003	.016	3.436	.064			
	297	.006	.003	.468	.494			
	299	.003	.000	1.507	.220			
D20S156						3.7	5	.59
	181	.007	.000	1.097	.295			
	183	.149	.130	.297	.586			
	185	.518	.506	.050	.822			
	187	.298	.351	1.282	.257			
	189	.021	.013	.380	.538			
	191	.007	.000	1.097	.295			
D20S892						20.8	12	.05
	199	.006	.000	2.980	.084			
	201	.006	.000	2.980	.084			
	203	.106	.076	2.110	.146			
	205	.017	.036	2.622	.105			
	207	.011	.044	7.635	.006			
	209	.299	.284	.199	.656			
	211	.060	.047	.656	.418			
	213	.121	.130	.151	.698			
	215	.313	.307	.030	.863			
	217	.043	.057	.772	.380			
	219	.014	.013	.025	.876			
	221	.003	.003	.005	.944			
	223	.000	.003	1.292	.256			
D20S846						4.0	8	.85
	269	.349	.318	.832	.362			
	271	.009	.005	.300	.584			
	273	.261	.294	.991	.320			
	275	.071	.086	.565	.452			
	277	.017	.013	.202	.653			
	279	.153	.159	.041	.839			
	281	.102	.091	.261	.610			
	283	.034	.034	.000	.986			
	285	.003	.000	1.477	.224			

LRS, likelihood ratio statistics; DR, degrees of freedom.

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American Journal of Medical Genetics Part B (Neuropsychiatric Genetics) 121B:7–13 (2003)

Association of a Haplotype in the Serotonin 5-HT4 Receptor Gene (*HTR4*) With Japanese Schizophrenia

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The serotonin 5-HT_4 receptor (5-HT_4) is implicated in cognitive function, of which impairment is hypothesized as one of the core disturbances of schizophrenia. Linkage analysis shows that 5q33.2, in which HTR4 is located, is schizophrenia-susceptibility loci. We therefore hypothesized that variation in the 5-HT_4 receptor gene (*HTR4*) modifies genetic susceptibility to schizophrenia. HTR4 coding regions and introns that include the branch sites of HTR4 were investigated in 96 unrelated Japanese schizophrenics using denaturing high-performance liquid chromatography analysis. One silent single nucleotide polymorphism (SNP) within the coding region and six intronic SNPs were detected. 353 + 6G > A was located in the branch site that could be effect to RNA splicing. None of the four SNPs, in which rare-allele frequencies were more than 10% was associated with 189 schizophrenics, in comparison to 299 controls. However, a highly significant association between schizophrenia and haplotype A-T (OR = 0.13 [0.03–0.58]) was detected. These findings suggest that haplotype A-T itself may inhibit the occurrence of schizophrenia, or that another susceptible genetic variants may exist within linkage disequilibrium. © 2003 Wiley-Liss, Inc.

KEY WORDS: **schizophrenia; association study; haplotype analysis; serotonin 5-HT4 receptor single nucleotide polymorphism**

INTRODUCTION

Cognitive dysfunction has been regarded as an fundamental problem of schizophrenia [Andreasen et al., 1996; Andreasen et al., 1999; Elvevag and Goldberg, 2000], therefore molecular mechanisms of cognitive function may have an important role in the pathophysiology of schizophrenia. The 5-HT_4 receptor (5-HT_4) is widely believed to be one of the important neurotransmission receptor, which participates in cognitive function [Buhot, 1997; Buhot et al., 2000].

Stimulation of cAMP, which is triggered by the activation of 5-HT_4, plays an important role of the hippocampal long-term potentiation (LTP) [Otmakhova et al., 2000] that is the basic cellular mechanism underlying learning and memory [Bliss and Collingridge, 1993]. Added to this, the stimulation of cAMP also induces facilitation of acetylcholine release that is of importance for the mechanisms in memory functions. Augmentation of LTP formation induced by a 5-HT_4 agonist SC 53116 was prevented by selective 5-HT_4 antagonist GR 113808, as well as by scopolamine [Matsumoto et al., 2001]. From behavioral studies in rats, 5-HT_4 agonists and antagonists modulate the cognitive process in regard to short-term and long-term memory [Letty et al., 1997].

Genome wide linkage analysis confirms that there is strong support, from multiple studies, for schizophrenia-susceptibility loci on 5q33.2, [Kendler et al., 2000; Levinson et al., 2000; Gurling et al., 2001] in which HTR4 located in (5q31–5q33).

Furthermore, HTR4 is composed of five exons and produces nine splice variants, 5-$HT_{4(a)}$, 5-$HT_{4(b)}$, 5-$HT_{4(c)}$, 5-$HT_{4(d)}$, 5-$HT_{4(h)}$, 5-$HT_{4(gef)}$, 5-$HT_{4(n)}$ (Fig. 1) [Blondel et al., 1998; Claeysen et al., 1999; Bender et al., 2000; Vilaro et al., 2002]. These variants alter the mRNA expression levels at each brain area and have different pharmacological profiles, particularly in the hippocampus,

Grant sponsor: The Ministry of Education, Culture, Sports, Science and Technology; Grant sponsor: The Ministry of Health, Labor and Welfare.

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Received 24 September 2002; Accepted 7 April 2003
DOI 10.1002/ajmg.b.20060