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

Chihiro Kakiuchi[1], Kazuya Iwamoto[1], Mizuho Ishiwata[1], Miki Bundo[1], Takaoki Kasahara[1], Ichiro Kusumi[2], Takahiro Tsujita[3], Yuji Okazaki[4], Shinichiro Nanko[5], Hiroshi Kunugi[6], Tsukasa Sasaki[7] & Tadafumi Kato[1]

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.

[1]Laboratory for Molecular Dynamics of Mental Disorders, Brain Science Institute, RIKEN, Wako-shi, Saitama 351-0198, Japan. [2]Department of Psychiatry, Hokkaido University, Sapporo-shi, Hokkaido 060-8648, Japan. [3]Department of Psychiatry, Nagasaki University, Nagasaki-shi, Nagasaki 852-8523, Japan. [4]Department of Psychiatry, Mie University, Tsu-shi, Mie 514-8507, Japan. [5]Department of Psychiatry, Teikyo University School of Medicine, Itabashi-ku, Tokyo 173-8605, Japan. [6]Department of Mental Disorder Research, National Institute of Neuroscience, Kodaira-shi, Tokyo 187-8502, Japan. [7]Department of Psychiatry, Health Service Center, University of Tokyo, Bunkyo-ku, Tokyo 113-0033, Japan. Correspondence should be addressed to T. Kato (kato@brain.riken.go.jp).

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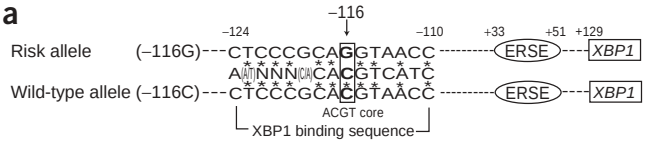


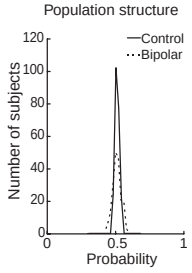
b

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

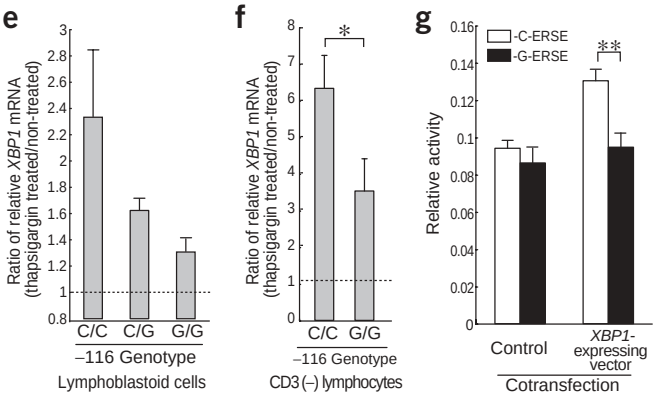


Figure 1 –116C→G polymorphism in *XBP1* is a risk factor for bipolar disorder. (**a**) –116C→G abolishes the ACGT core sequence. Numbers indicate the nucleotide positions from the transcription start site. (**b**) Association of the –116C→G polymorphism with bipolar disorder in a case-control study. (**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*

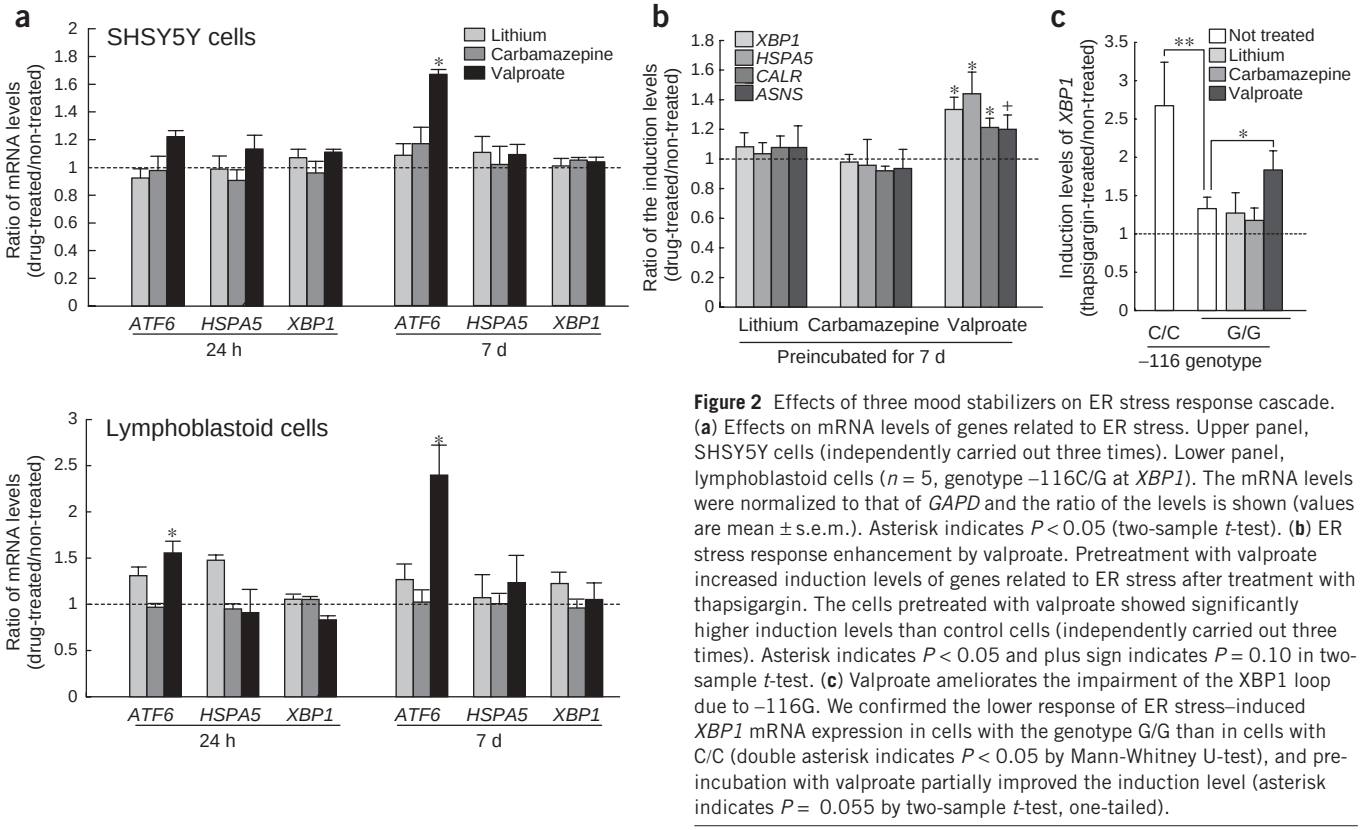


Figure 2 Effects of three mood stabilizers on ER stress response cascade. (**a**) Effects on mRNA levels of genes related to ER stress. Upper panel, SHSY5Y cells (independently carried out three times). Lower panel, lymphoblastoid cells ($n = 5$, genotype –116C/G at *XBP1*). The mRNA levels were normalized 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*U1 (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*1/*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.

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COMPETING INTERESTS STATEMENT

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

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