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ORIGINAL ARTICLE

Association analysis of *HSP90B1* with bipolar disorder

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Abstract Pathophysiological role of endoplasmic reticulum (ER) stress response signaling has been suggested for bipolar disorder. The goal of this study was to test the genetic association between bipolar disorder and an ER chaperone gene, *HSP90B1* (*GRP94*/gp96), which is located on a candidate locus, 12q23.3. We tested the genetic association between bipolar disorder and *HSP90B1* by case-control studies in two independent Japanese sample sets and by a transmission disequilibrium test (TDT) in NIMH Genetics initiative bipolar trio samples (NIMH trios). We also performed gene expression analysis of *HSP90B1* in lymphoblastoid cells. Among the 11 SNPs tested, rs17034977 showed significant association in both Japanese sample sets. The frequency of the SNP was lower in NIMH samples than in Japanese samples and there was no significant association in NIMH trios. Gene expression analysis of *HSP90B1* in lymphoblastoid cells suggested a possible relationship between the associated SNP and mRNA levels. *HSP90B1* may have a pathophysiological role in bipolar disorder in the Japanese population, though further study will be needed to understand the underlying functional mechanisms.

Keywords Bipolar disorder · *HSP90B1*/*GRP94*/*gp96* · Association study · Evi12 · Endoplasmic reticulum stress · Retrovirus

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Introduction

Although the contribution of genetic factors to bipolar disorder has been evidenced by family, twin and adoption studies, the genetic risk factors for the illness are still controversial (Kato 2007). We previously suggested that endoplasmic reticulum (ER) stress response signaling is one of the candidate cascades that could be related to pathology of the illness (Kakiuchi et al 2003, 2005, 2006).

ER is a protein folding system. When unfolded proteins accumulate in ER, which could occur for a number of reasons, the ER stress response begins. ER stress response consists of four signaling cascades: (1) induction of ER chaperone such as *HSPA5* (also named as *GRP78* or *BiP*), *HSP90B1* (*GRP94* or *gp96*), *CALR* (*Calreticulin*), etc., which promotes the folding of unfolded proteins, (2) inhibition of protein synthesis, (3) induction of an ER-associated degradation pathway, which promotes the processing of unfolded proteins, and (4) induction of apoptosis when this system cannot process the unfolded proteins (Schroder and Kaufman 2005; Yoshida 2004).

When ER chaperones are consumed to fold unfolded proteins, HSPA5 is dissociated from ATF6 protein on the ER membrane, which causes cleavage of ATF6. Cleaved ATF6 protein induces the expression of ER chaperones and *XBP1*. In parallel, dissociation of HSPA5 from IRE1 protein on the ER membrane causes dimerization of IRE1, which splices *XBP1* mRNA. The spliced *XBP1* mRNA encodes an active form of XBP1 that strongly induces the expression of target genes such as ER chaperones (Yoshida 2004).

Several lines of evidence have suggested that ER stress related genes are involved in the pathophysiology of affective illness including bipolar disorder. The first evidence was detection of ER chaperones such as *HSPA5*, *HSP90B1* and *CALR* as target genes of a mood stabilizer, valproate, using differential display (Wang et al. 1999). Subsequently, increased expression of ER chaperones in the temporal cortex of depressed subjects who died by suicide was reported (Bown et al. 2000). One microarray study using postmortem brain samples showed altered expression of *HSP90B1* (*gp96*) in the bipolar disorder patients (Jurata et al. 2004). The anti-malarial drug mefloquine, which is known to cause psychiatric symptoms including bipolar disorder in susceptible individuals (Even et al. 2001), is reported to induce ER stress in rat neurons (Dow et al. 2003, 2005). Methamphetamine (MAP), a psychostimulant causing a manic state, is known to induce ER chaperone genes in the mouse brain (Jayanthi et al. 2004). Foot shock stress or increase of serotonergic neurotransmission by the treatment with 5-hydroxy-l-tryptophan and clorgyline caused enhancement of *XBP1* splicing in rats (Toda et al. 2006). Two common mood stabilizers, valproate and lithium, have neuroprotective effects by inducing ER chaperones (Hiroi et al. 2005; Kim et al. 2005; Wang et al. 2003). Valproate also enhanced the ER stress response to thapsigargin (Kakiuchi et al. 2003).

We previously showed by DNA microarray analysis that *XBP1* and *HSPA5* are down-regulated in the lymphoblastoid (LB) cells of monozygotic twins with bipolar disorder compared with healthy co-twins (Kakiuchi et al. 2003). Induction of *XBP1* and *HSPA5* mRNA by thapsigargin was reduced in the patients' cell lines. Recently, So et al. (2007) also reported that induction of XBP1 and CHOP by thapsigargin and tunicamycin was attenuated in the lymphoblastoid cells derived from patients with bipolar disorder. Although we also reported that a functional polymorphism of *XBP1* was associated with bipolar disorder, the genetic association was not replicated in Caucasian samples and Taiwanese samples (Cichon et al. 2004; Hou et al. 2004). We further performed a genetic association study of *HSPA5* and demonstrated nominal association on rs16927997 in the Japanese population (Kakiuchi et al. 2005). However, the association was not replicated in an independent sample set in that study and no association was found in NIMH trio samples. The other ER stress related genes ATF4/ATF5 were not found to be associated with bipolar disorder (Kakiuchi et al. 2007). Thus, association of the ER stress related genes with bipolar disorder is still controversial.

In this study, we investigated a potential genetic association of *HSP90B1*, the other ER chaperone gene, with bipolar disorder. *HSP90B1* expression is induced by treatment with valproate and lithium (Shao et al. 2006) and its expression is altered in the postmortem brain of bipolar disorder patients (Bown et al. 2000). The *HSP90B1* gene is located on 12q23.3. Many studies have shown the significant linkage of 12q23-24 with bipolar disorder (Curtis et al. 2003; Dawson et al. 1995; Detera-Wadleigh et al. 1999; Ekholm et al. 2003; Ewald et al. 1998; Green et al. 2005; Maziade et al. 2005; Morissette et al. 1999). We also performed expression analysis in LB cells derived from patients and controls.

Materials and methods

Subjects

For the case control study in the Japanese population, a total of 449 patients with bipolar disorder (50.2 ± 13.4 years old, 216 males and 233 females, 318 with bipolar I disorder, 126 with bipolar II disorder and 5 with schizoaffective disorder, bipolar type) and 543 controls (43.4 ± 15.0 years old, 269 males and 274 females) were analyzed. These samples are similar to those previously

described and included two independently collected sample sets (Kakiuchi et al. 2005). One set consisted of samples that were not age- and sex-matched (MDMD samples), including 212 patients with bipolar disorder (49.0 ± 13.5 years old, 87 males and 125 females, 152 with bipolar I and 60 with bipolar II disorder) and 304 controls (37.1 ± 14.8 years old, 149 males and 155 females). The other set was age- and sex-matched samples ("MPS samples"), which included 237 patients with bipolar disorder (51.2 ± 13.2 years old, 129 males and 108 females, 166 with bipolar I, 66 with bipolar II, and 5 with schizoaffective disorder bipolar type) and 239 controls (51.5 ± 10.7 years old, 120 males and 119 females). To test the specificity of the findings, 227 patients with schizophrenia (46.0 ± 14.9 years old, 130 males and 97 females) were also examined. They were diagnosed according to the DSM-IV criteria (American Psychiatric Association) by the consensus of at least two psychiatrists. Structured interviews were not used for the diagnosis, except for use of a structured interview, the Mini-International Neuropsychiatric Interview (M.I.N.I.) (Sheehan et al. 1998), for recently recruited 17 patients. Controls were selected from students, nurses, office workers, and doctors in participating institutes, and their friends. A senior psychiatrist, one of authors, interviewed them and they did not have major mental disorders.

In Japanese, no significant population stratification has been repeatedly reported in several studies including a part of our samples (Arinami et al. 2005; Kakiuchi et al. 2003; Yamada et al. 2004). For the transmission disequilibrium test, we analyzed a total of 238 trio samples from NIMH Genetics Initiative Pedigrees. Only one trio was obtained from one family. The criteria, by which the trio was selected from a pedigree, were as follows: (1) DNA is available for parents and the proband, (2) if multiple complete trios were found in one pedigree, the trio with a younger generation was selected, and (3) if multiple trios were available in one generation, an elder sibling was selected as the proband. Data and biomaterials of the 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: Indiana University, Indianapolis, IN, U01 MH46282, J. Nurnberger, M. Miller, and E. Bowman; Washington University, St. Louis, MO, U01 MH46280, T. Reich, A. Goate, and J. Rice; Johns Hopkins University, Baltimore, MD U01 MH46274, J. R. DePaulo, Jr., S. Simpson, and C. Stine; NIMH Intramural Research Program, Clinical Neurogenetics Branch, Bethesda, MD, E. Gershon, D. Kazuba, and E. Maxwell. In these pedigrees, linkage with 12q23-q24 was reported (Detera-Wadleigh et al. 1999). For the mRNA expression analysis, we examined the mRNA expression level of *HSP90B1* in LB cells derived from 23 patients (51.3 ± 11.9 years old, 16 males and 7 females) and 19 controls (49.0 ± 13.1 years old, 12 males and 7 females). For the Southern blotting analysis, we used genomic DNA derived from LB cells of 53 patients who were also included in the Japanese case-control analysis. Written informed consent was obtained from all subjects. The Ethics Committees of the Brain Science Institute (RIKEN) and participating institutes approved the study.

Genetic association studies

In total, seven SNPs (rs1165678, rs1165681, rs1165687, rs1882019, rs17034977, rs703657 and rs2293618) were selected according to the LD Map Database on SNP-browser^{TM} of Applied Biosystems (Applied Biosystems; Foster city, CA, USA) (Fig. 1). These SNPs were selected because their TaqMan probes were commercially available

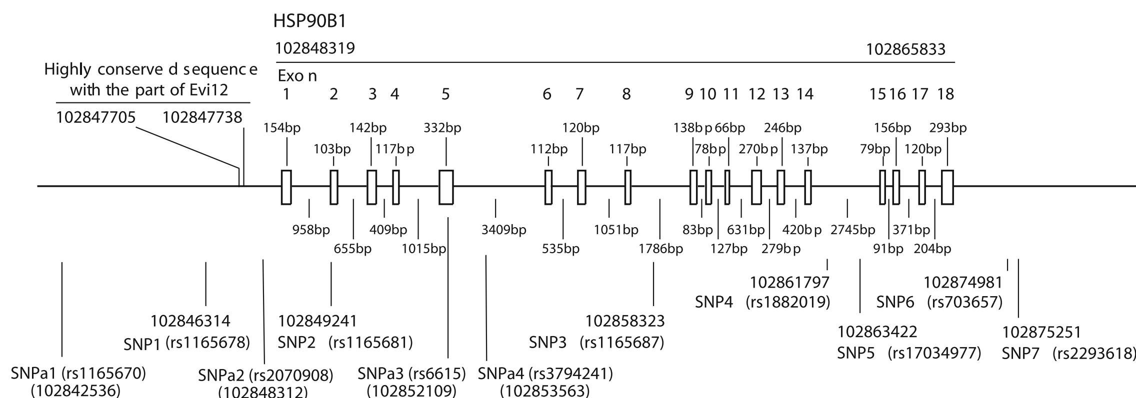

Fig. 1 The genomic structure of *HSP90B1*. *HSP90B1* consists of 18 exons. A highly conserved 33-bp sequence is observed in the upstream region. *Number* indicates the position in the UCSC Human Genome Browser, March 2006

and they are polymorphic in Japanese. Based on the results obtained, we genotyped 4 additional SNPs (rs1165670, rs2070908, rs6615 and rs3794241). They were selected from the database because they have similar frequency and are supposed to be linked with rs17034977. Genotyping was performed using commercially available TaqMan probes and ABI7900HT according to the protocol recommended by the manufacturer (Applied Biosystems). Assessment of linkage disequilibrium (LD) patterns by the standardized disequilibrium coefficient (D') and squared correlation coefficient (r^2), analysis of haplotypic distribution and frequencies, and haplotype TDT analysis were performed using the COCAPHASE and TDTPHASE programs (http://www.portal.litbio.org/Registered/Option/unphased.html).

Sequencing

To identify the possible functional polymorphisms that are linked to the haplotype associated with bipolar disorder, we sequenced the upstream region, 5′-untraslated region (UTR), exon14, exon17 and exon18, in which the existence of missense polymorphisms has been shown in UCSC Human Genome Browser, March 2006 (http://www.genome.ucsc.edu/), and 3′-UTR of *HSP90B1* in 44 patients with bipolar disorder. We also sequenced around the highly conserved region with Evi12. Primer sequences are as follows: promotor region and 5′-UTR, forward 5′-CTGACCAATCGGAAGGAG and reverse 5′-ATCACTCACCGAAGGTCA; exon14, forward 5′-GACAGTTGAAAGACAATTGCTC and reverse 5′-CGTAAACTTGTTGTCTACCT; exon17, forward 5′-GAAAGAATGCTTCGCCTCAG and reverse 5′-ACTCTGTCCTAACTTTGCCAGAAC; exon18 and 3′-UTR, forward 5′-CATGCACAAACCTGTGAG and reverse 5′-TCAGCCAAGTATTAGGTAGT; around Evi12, forward 5′-GCACATATCTCTACTGTAATCAGAACGAG and reverse 5′-CCTGGGGTTTCGTTGGAAGTG.

Quantification of mRNA levels

We examined the mRNA expression level of *HSP90B1* in LB cells. We transformed lymphocytes from peripheral blood by Epstein–Barr virus and cultured LB cells in the medium of RPMI1640 (Sigma; Saint Louis, Missouri, USA) containing 10% fetal bovine serum (FBS) as the same methods described previously (Kato et al. 2003). Then, LB cells were collected into TRIZOL reagents (Invitrogen; San Diego, CA, USA). Total RNA was extracted and single strand cDNA was synthesized using oligo (dT) primer and SuperScript II reverse transcriptase (Invitrogen) by the same methods described previously (Kakiuchi et al. 2003). For the quantification of mRNA levels, real-time quantitative PCR using TaqMan chemistry was performed according to the manufacturer's protocol (Applied Biosystems). For quantitative PCR, ABI7900HT was used and the relative ratios to *GAPDH* were calculated. The experiments were performed in quadliplicate. Primer and probe mixture of *HSP90B1* and *GAPDH* was obtained from assay-on-demand products (Applied Biosystems). *T* test was used for comparison of the expression levels between controls and patients in LB cells.

Southern blotting

Southern blotting analysis was performed to examine the insertion/deletion mutations around the *HSP90B1* gene. Ten micrograms of genomic DNA derived from LB cells was digested by EcoRV (Takara; Otsu, Shiga, Japan) and run on a 0.8% agarose gel. After transferring to nylon membrane, hybridization was performed using two kinds of digoxigenin-labeled probes. Probes were made as follows. PCR-products were cloned into pCR-2.1-TOPO Vector (Invitrogen), and then internally labeled with digoxigenin using a PCR DIG Probe Synthesis Kit (Roche, Germany) according to the manufacturer's protocol. Primers are as follows: forward, ATGGGACCTGACCCAAGT and reverse, GTAATGTCCCACACAATTTAGGAG, which detect 4642 bp fragments on the upstream region of *HSP90B1* (chromosome 12, 102839756–102844427, UCSC Human Genome Browser, March 2006 Assembly); forward, CACGAATCCTCATTGGGT and reverse, CAGTTAGATGATGTTGCTCTCT, which detect 6050 bp fragments (chromosome 12, 102844428–102850477). Hybridization of the probes was detected by LAS-3000 luminoimage analyzer (Fujifilm; Tokyo, Japan) using a part of DIG High Prime DNA Labeling and Detection Starter Kit II (Roche).

Statistical analysis

For statistical analysis, Chi-square test and Mann–Whitney *U* test were used. The standardized disequilibrium coefficient (D') and squared correlation coefficient (r^2) was calculated using the COCAPHASE program. The standardized disequilibrium coefficient (D') and squared correlation coefficient (r^2) in the trio samples was calculated using the TDTPHASE program. For the association analysis, P values obtained by the Chi-square test before the Bonferroni correction were shown.

Results

Case control study for bipolar disorder in Japanese samples

The structure of *HSP90B1* is shown in Fig. 1. The results of association analysis of single SNPs are shown in Table 1. rs17034977 was significantly associated with bipolar disorder in both sample sets, "MDMD samples" and "MPS samples", although they are no more significant after the Bonferroni correction. The allelic association of bipolar disorder with rs17034977 was statistically significant even after the Bonferroni correction in the combined samples ($P = 0.005$ for genotype and $P = 0.001$ for allele. After Bonferroni correction considering seven markers and two comparisons, $P = 0.07$ and $P = 0.014$, respectively). There were no significant relationship between lithium response and the rs17034977 genotypes in the patients whose clinical information on lithium response was available (data not shown).

Because only one SNP was associated with bipolar disorder in a case control study in Japanese, we genotyped four additional SNPs (rs1165670, rs2070908, rs6615 and rs3794241) which have similar frequency and are supposed to be linked with rs17034977. None of these SNPs (SNPs a1–a4) were significantly associated with bipolar disorder (Table 1). Linkage disequilibrium by the D' and r^2 in the control subjects is shown in Fig. 2. Because SNPs 3–7 are in the haploptype block, we performed the haplotype analysis of SNPs 3–7 (Table 2). There are three haplotypes, CAATT, CGAAC, and CGCTT, of which, CGCTT was significantly associated with bipolar disorder (global significance by random permutation test [10,000 times] using COCAPHASE program was $P = 0.0026$). This haplptype is determined by the SNP5, and thus SNP5 can be regarded as the tag SNP in this haplotype block.

Table 1 The result of case-control studies in Japanese population

		MDMD samples									MPS samples									Total samples							
		Genotype			HWE	p-value	Allele		p-value		Genotype			HWE	p-value	Allele		p-value		Genotype			HWE	p-value	Allele		p-value
rs1165670		A	A/G	G	Hardy	p	A	G	p		A	A/G	G	Hardy	p	A	G	p		A	A/G	G	Hardy	p	A	G	p
(SNPa1)	CT	150	126	27	0.941		426	180		CT	112	105	22	0.712		329	149		CT	262	231	49	0.851		755	329	
	BP	100	91	18	0.672	0.908	291	127	0.815	BP	103	106	28	0.927	0.579	312	162	0.323	BP	203	197	46	0.860	0.618	603	289	0.328
rs1165678		A	A/G	G	Hardy	p	A	G	p		A	A/G	G	Hardy	p	A	G	p		A	A/G	G	Hardy	p	A	G	p
(SNP1)	CT	206	83	15	0.088		495	113		CT	168	64	7	0.763		400	78		CT	374	147	22	0.123		895	191	
	BP	140	64	8	0.839	0.6700	344	80	0.9089	BP	167	60	10	0.133	0.7214	394	80	0.8165	BP	307	124	18	0.227	0.9817	738	160	0.8937
																			SZ	163	54	10	0.053	0.6356	380	74	0.5415
rs2070908		C	C/G	G	Hardy	p	C	G	p		C	C/G	G	Hardy	p	C	G	p		C	C/G	G	Hardy	p	C	G	p
(SNPa2)	CT	84	144	75	0.397		312	294		CT	66	114	59	0.485		246	232		CT	150	258	134	0.272		558	526	
	BP	69	102	38	0.977	0.164	240	178	0.061	BP	72	111	54	0.373	0.773	255	219	0.471	BP	141	213	92	0.484	0.214	495	397	0.075
rs1165681		C	C/T	T	Hardy	p	C	T	p		C	C/T	T	Hardy	p	C	T	p		C	C/T	T	Hardy	p	C	T	p
(SNP2)	CT	75	143	86	0.312		293	315		CT	59	114	66	0.485		232	246		CT	134	257	152	0.222		525	561	
	BP	38	104	70	0.953	0.1632	180	244	0.0687	BP	54	109	74	0.257	0.6763	217	257	0.3945	BP	92	213	144	0.417	0.1957	397	501	0.0662
																			SZ	57	114	56	0.947	0.6237	228	226	0.5015
rs6615		C	C/T	T	Hardy	p	C	T	p		C	C/T	T	Hardy	p	C	T	p		C	C/T	T	Hardy	p	C	T	p
(SNPa3)	CT	150	129	24	0.607		429	177		CT	111	107	21	0.503		329	149		CT	261	236	45	0.412		758	326	
	BP	95	91	23	0.863	0.421	281	137	0.224	BP	114	95	28	0.237	0.418	323	151	0.820	BP	209	186	51	0.329	0.253	604	288	0.290
rs3794241		C	C/T	T	Hardy	p	C	T	p		C	C/T	T	Hardy	p	C	T	p		C	C/T	T	Hardy	p	C	T	p
(SNPa4)	CT	176	117	10	0.072		469	137		CT	154	74	11	0.584		382	96		CT	330	191	21	0.304		851	233	
	BP	111	89	9	0.088	0.505	311	107	0.270	BP	145	85	7	0.189	0.384	375	99	0.759	BP	256	174	16	0.038	0.473	686	206	0.395
rs1165687		A	A/C	C	Hardy	p	A	C	p		A	A/C	C	Hardy	p	A	C	p		A	A/C	C	Hardy	p	A	C	p
(SNP3)	CT	0	6	298	0.862		6	602		CT	0	5	234	0.870		5	473		CT	0	11	532	0.812		11	1075	
	BP	0	5	207	0.862	0.7659	5	419	0.7671	BP	0	3	234	0.922	0.4832	3	471	0.4851	BP	0	8	441	0.849	0.7801	8	890	0.7812
rs1882019		A	A/G	G	Hardy	p	A	G	p		A	A/G	G	Hardy	p	A	G	p		A	A/G	G	Hardy	p	A	G	p
(SNP4)	CT	34	132	138	0.774		200	408		CT	26	105	108	0.949		157	321		CT	60	237	246	0.797		357	729	
	BP	27	98	87	0.942	0.6009	152	272	0.3247	BP	35	91	111	0.027	0.3072	161	313	0.7139	BP	62	189	198	0.121	0.4192	313	585	0.3527
																			SZ	29	94	104	0.289	0.7379	152	302	0.8173
rs17034977		A	A/C	C	Hardy	p	A	C	p		A	A/C	C	Hardy	p	A	C	p		A	A/C	C	Hardy	p	A	C	p
(SNP5)	CT	189	96	19	0.158		474	134		CT	142	85	12	0.875		369	109		CT	331	181	31	0.346		843	243	
	BP	150	55	7	0.485	0.0858	355	69	**0.0219**	BP	165	65	7	0.845	0.0579	395	79	**0.0174**	BP	315	120	14	0.536	**0.0056**	750	148	**0.0010**
																			SZ	153	64	10	0.327	0.2349	370	84	0.0901
rs703657		A	A/T	T	Hardy	p	A	T	p		A	A/T	T	Hardy	p	A	T	p		A	A/T	T	Hardy	p	A	T	p
(SNP6)	CT	60	149	95	0.909		269	339		CT	40	128	71	0.167		208	270		CT	100	277	166	0.407		477	609	
	BP	44	110	58	0.538	0.6352	198	226	0.4357	BP	57	114	66	0.573	0.1378	228	246	0.1556	BP	101	224	124	0.993	0.2451	426	472	0.1175
																			SZ	56	101	70	0.108	0.1065	213	241	0.2814
rs2293618		C	C/T	T	Hardy	p	C	T	p		C	C/T	T	Hardy	p	C	T	p		C	C/T	T	Hardy	p	C	T	p
(SNP7)	CT	63	149	92	0.852		275	333		CT	43	127	69	0.243		213	265		CT	106	276	161	0.528		488	598	
	BP	46	111	55	0.475	0.5622	203	221	0.4015	BP	61	110	66	0.272	0.1112	232	242	0.1752	BP	107	221	121	0.757	0.2365	435	463	0.1192
																			SZ	58	102	67	0.132	0.1446	218	236	0.2683

For the SNPs a1-a4, the number of subjects were 446 for bipolar disorder and 542 for controls.

r2 \ D'		SNPa1 rs1165670	SNP1 rs1165678	SNPa2 rs2070908	SNP2 rs1165681	SNPa3 rs6615	SNPa4 rs3794241	SNP3 rs1165687	SNP4 rs1882019	SNP5 rs17034977	SNP6 rs703657	SNP7 rs2293618
SNPa1	rs1165670		1	1	1	1	0.1005	1	0.6398	0.6569	0.68	0.6687
SNP1	rs1165678	0.0932		0.9849	1	0.9599	0.6009	0.8745	0.8724	1	0.8478	0.9485
SNPa2	rs2070908	0.4623	0.1956		1	1	0.2524	0.7385	0.6904	0.7107	0.1034	0.07843
SNP2	rs1165681	0.464	0.2009	0.9963		1	0.2554	0.7377	0.6892	0.7117	0.09883	0.07412
SNPa3	rs6615	0.1874	0.08476	0.4054	0.4039		0.3821	1	0.8698	0.9051	0.8203	0.8272
SNPa4	rs3794241	0.001204	0.02115	0.01645	0.01677	0.09293		0.044	0.3828	0.3635	0.4606	0.4473
SNP3	rs1165687	0.004467	0.03665	0.005271	0.00524	0.004409	7.25E-05		1	1	1	1
SNP4	rs1882019	0.08686	0.07927	0.2188	0.2172	0.6682	0.0824	0.004992		0.979	1	1
SNP5	rs17034977	0.05433	0.0618	0.1548	0.1558	0.1018	0.01045	0.002962	0.1349		1	1
SNP6	rs703657	0.2564	0.1956	0.008908	0.008173	0.2274	0.04566	0.008056	0.3827	0.2271		1
SNP7	rs2293618	0.238	0.235	0.005343	0.004789	0.241	0.04485	0.01252	0.3987	0.2366	0.9597	

D' larger than 0.9 was shown in shaded cells.

Fig. 2 Intermarker linkage disequilibrium pattern in Japanese samples. The standardized disequilibrium coefficient (D') and squared correlation coefficient (r^2) was calculated for total controls using the COCAPHASE program. D' larger than 0.9 was shown in *shaded* cells

Table 2 Haplotype analysis of *HSP90B1* in Japanese BP samples

Haplotype	MDMD samples			MPS samples			Total samples		
(SNPs3–7)	BP	CT	*P*	BP	CT	*P*	BP	CT	*P*
1. C–A–A–T–T	150 (0.36)	197 (0.33)	0.27	158 (0.34)	155 (0.33)	0.72	308 (0.35)	352 (0.33)	0.31
2. C–G–A–A–C	196 (0.47)	266 (0.44)	0.35	224 (0.48)	205 (0.43)	0.15	420 (0.48)	471 (0.44)	0.094
3. C–G–C–T–T	66 (0.16)	133 (0.22)	**0.012**	78 (0.16)	106 (0.22)	**0.027**	144 (0.16)	239 (0.22)	**0.00094**
	Global *P* = 0.037			Global *P* = 0.071			**Global *P* = 0.0026**		

Global significance was calculated by a random permutation test (10,000 times) using the COCAPHASE program

Only haplotypes that were verified at least once were analyzed

Significant *P*-values are shown in bold

TDT analysis for bipolar disorder in NIMH trios

We examined the association in other ethnicities by TDT analysis of NIMH bipolar trio samples, which are mainly derived from Caucasians. No significant over–transmission was observed in any SNPs (Table 3). rs17034977 was not significantly associated. Linkage disequilibrium by the D' and r^2 in the transmitted allele is shown in Fig. 3.

Case control study for schizophrenia in Japanese samples

We further performed association analysis of the *HSP90B1* locus for schizophrenia in Japanese samples because schizophrenia and bipolar disorder are known to share a common genetic background. We genotyped six of the SNPs (SNP1, 2, 4, 5, 6, and 7). The genotype and allele frequencies were compared with the total Japanese controls used for the analysis of bipolar disorder. No significant association was observed in single SNPs (Table 1). However, the result should be treated with caution, because there was a significant difference of gender between patients with schizophrenia and controls ($P < 0.05$).

HSP90B1 mRNA levels in lymphoblastoid cells

We examined the mRNA expression level of *HSP90B1* in LB cells derived from 22 bipolar disorder patients and 19 controls. There were no statistically significant differences between controls and patients in relative expression levels of *HSP90B1* to *GAPDH* in the cells ($P = 0.468$, Mann–Whitney *U* test; Fig. 4).

The carrier of C allele of SNP5 (rs17034977) had significantly lower expression levels of *HSP90B1* ($n = 25$, 0.029 ± 0.008, mean ± SD) compared with those carrying homozygous A alleles ($n = 17$, 0.036 ± 0.009, $P = 0.035$, Mann–Whitney *U* test; Fig. 5).

Search for possible functional polymorphisms

We tried to identify the functional SNPs linking to the haplotype responsible for the association with bipolar disorder and the alteration of *HSP90B1* expression level. Public databases were searched and 44 DNA samples of bipolar disorder patients were sequenced. Putative functional polymorphisms, such as variation within the promoter region, polymorphisms altering amino acid

Table 3 The result of TDT analysis in NIMH trio samples

	Allele	Transmitted	Not-transmitted	*P* value
rs1165670	A	352 (0.7395)	342 (0.7185)	0.466
(SNPa1)	G	124 (0.2605)	134 (0.2815)	
rs1165678	A	288 (0.6025)	293 (0.613)	0.741
(SNP1)	G	190 (0.3975)	185 (0.387)	
rs2070908	C	318 (0.6681)	316 (0.6639)	0.891
(SNPa2)	G	158 (0.3319)	160 (0.3361)	
rs1165681	C	159 (0.3326)	163 (0.341)	0.784
(SNP2)	T	319 (0.6674)	315 (0.659)	
rs6615	C	416 (0.8739)	419 (0.8803)	0.767
(SNPa3)	T	60 (0.1261)	57 (0.1197)	
rs3794241	C	422 (0.8866)	432 (0.9076)	0.286
(SNPa4)	T	54 (0.1134)	44 (0.09244)	
rs1165687	A	231 (0.4833)	236 (0.4937)	0.746
(SNP3)	C	247 (0.5167)	242 (0.5063)	
rs1882019	A	49 (0.1025)	49 (0.1025)	1.000
(SNP4)	G	429 (0.8975)	429 (0.8975)	
rs17034977	A	441 (0.9226)	437 (0.9142)	0.636
(SNP5)	C	37 (0.07741)	41 (0.08577)	
rs703657	A	160 (0.3347)	152 (0.318)	0.581
(SNP6)	T	318 (0.6653)	326 (0.682)	
rs2293618	C	391 (0.818)	387 (0.8096)	0.740
(SNP7)	T	87 (0.182)	91 (0.1904)	

Number (frequency)

sequence, and those in untranslated regions (UTR), were searched for. In the non-coding region immediately upstream of the HSP90B1 gene, there is a conserved region. This region corresponds to the common virus integration site Evi12, the target of Cas-Br-M murine leukemia virus (Valk et al. 1999; van den Akker et al. 2005) (Fig. 1). This region was also sequenced. In the database of UCSC Human Genome Browser, March 2006 (http://www.genome.ucsc.edu/), a total of 13 SNPs were suggested to exist in the 500-bp upstream region (rs3216180, rs17034921, rs2722187, rs2070908), 5′-UTR (rs17797090), 3′-UTR (rs1051185, rs11547719, rs2307842), or as missense polymorphisms in exon14 (rs3209749), exon17 (rs17034989, rs17159034, rs3037197) and exon18 (rs11547722) of *HSP90B1*. In the conserved region, there were two SNPs (rs17034917 and rs17034919).

In the 44 samples, rs2722187, rs17797090, rs17034989, rs17159034, rs11547722, rs1051185, rs11547719, rs2307842 and rs3209749 were not polymorphic. rs3216180 (mainly linked with haplotype 1 [CAATT]), rs17034921 (mainly linked with haplotype 1), rs2070908 (linked with rs1165681) and rs3037197 (mostly linked with rs1882019) were polymorphic but not linked to haplotype 3 [CGCTT]. rs17034917 was not polymorphic and rs17034919 was linked with rs17034921, but not linked with haplotype 3.

To rule out a possibility of large deletion or insertion affecting transcriptional activity, the upstream region spanning about 10 kb of *HSP90B1* gene was examined by Southern blotting. We did not observe an extra band, suggesting the insertion or deletion in the DNA of 53 patients with bipolar disorder derived from lymphoblastoid cells (data not shown).

Discussion

In this study, we showed the association of a SNP within the *HSP90B1* locus (SNP5, rs17034977) with bipolar disorder in a Japanese population. No significant association was observed in NIMH bipolar disorder trio samples and Japanese schizophrenia samples. We examined the mRNA expression levels of *HSP90B1*. There was no significant difference between controls and patients. The C allele of SNP5 (rs17034977) was significantly associated with lower expression levels of *HSP90B1*.

Although we searched for functional SNPs responsible for the association, no SNPs linked with the associated haplotype was identified. We did not find any deletion or insertion affecting the restriction fragment length examined by the Southern blot analysis in the upstream region. There

r2 \ D'		SNPa1 rs1165670	SNP1 rs1165678	SNPa2 rs2070908	SNP2 rs1165681	SNPa3 rs6615	SNPa4 rs3794241	SNP3 rs1165687	SNP4 rs1882019	SNP5 rs17034977	SNP6 rs703657	SNP7 rs2293618
SNPa1	rs1165670		1	0.9878	0.9878	0.9207	0.1477	0.896	0.8171	0.5583	0.853	0.7113
SNP1	rs1165678	0.23		1	1	0.953	0.6657	0.7142	0.8879	0.5211	0.6995	0.7354
SNPa2	rs2070908	0.6919	0.3243		1	1	0.3726	0.8762	0.9257	0.6976	0.6073	0.05459
SNP2	rs1165681	0.6853	0.3274	0.9906		1	0.3766	0.8774	0.9263	0.6965	0.6096	0.05071
SNPa3	rs6615	0.04307	0.08551	0.07166	0.07234		0.5894	0.6906	0.9533	1	0.8216	0.7325
SNPa4	rs3794241	0.0009835	0.03701	0.008824	0.009102	0.3082		0.5762	0.4899	1	0.02048	0.3641
SNP3	rs1165687	0.2644	0.3561	0.3567	0.361	0.06431	0.03972		1	1	1	0.9739
SNP4	rs1882019	0.02699	0.05905	0.04886	0.04939	0.723	0.2152	0.1073		1	1	1
SNP5	rs17034977	0.009254	0.01494	0.08256	0.08151	0.01216	0.01078	0.0788	0.009672		1	1
SNP6	rs703657	0.511	0.1602	0.3653	0.3716	0.04883	2.69E-05	0.469	0.05756	0.04227		1
SNP7	rs2293618	0.03986	0.07896	0.001341	0.001146	0.346	0.07587	0.1983	0.5131	0.3768	0.1122	

D' larger than 0.9 was shown in shaded cells.

Fig. 3 Intermarker linkage disequilibrium pattern in NIMH trio samples. The standardized disequilibrium coefficient (D') and squared correlation coefficient (r^2) was calculated for transmitted alleles using the TDTPHASE program. D' larger than 0.9 are shown in *shaded* cells

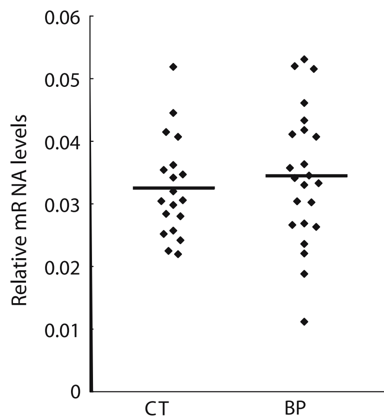

Fig. 4 *HSP90B1* mRNA levels in lymphoblastoid cells. *Y*-axis indicates the relative ratio of *HSP90B1* to GAPDH. No significant difference was observed between the cases of bipolar disorder (BP) and controls (CT)

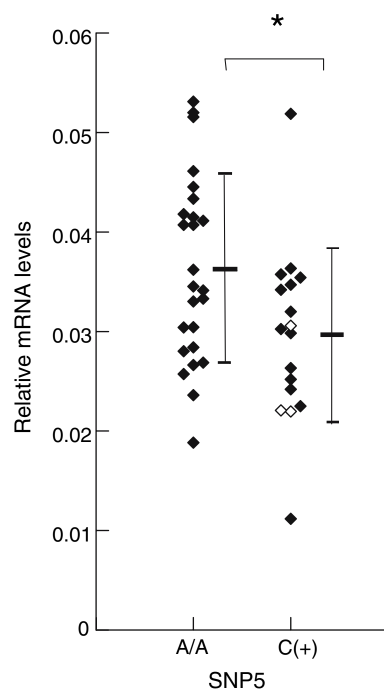

Fig. 5 Relationship between *HSP90B1* mRNA levels and SNP5 (rs17034977) in lymphoblastoid cells. *Y*-axis indicates the relative ratio of *HSP90B1* to *GAPDH*. A statistically significant difference of *HSP90B1* levels was observed between the homozygous A allele carriers and the C allele carriers. *Open square* indicates homozygote for the C allele of SNP 5 ($n = 3$). The *bars* show the average and standard deviations. $^{*}P = 0.035$, Mann–Whitney *U* test

is a conserved sequence in the upstream region of *HSP90B1*, corresponding to the common virus integration site Evi12, the target of Cas–Br–M murine leukemia virus in mice (van den Akker et al. 2005). In the case of mice, integration of retrovirus was postulated to affect the expression level of *HSP90B1* (Valk et al. 1999; van den Akker et al. 2005). There has been no evidence that supports the integration of retrovirus in this site in humans, and the present results also excluded such a possibility. Although the results of haplotype analysis, association analysis, mutation search, and mRNA analysis suggest that SNP5 itself might be important, it is still not known whether SNP5 itself affects the mRNA levels. Further studies will be needed to identify the genetic polymorphism affecting the mRNA levels.

Stratification analysis in Japanese samples including the part of the present samples did not show evidence suggesting the existence of population stratification (Yamada et al. 2004). Thus, it is unlikely that the observed difference is the artifact due to population stratification. However, it is possible that some control subjects might actually have bipolar disorder but have reported themselves as healthy, because patients with bipolar disorder often do not have insight into the illness. This might have confounded the results.

Lack of association in NIMH samples might suggest that the association between the SNP5 and bipolar disorder is ethnicity dependent. Indeed, it was reported that many associations between SNPs and expression levels were ethnicity dependent (Stranger et al. 2007).

A possibility that SNP5 is also associated with schizophrenia cannot be ruled out, because a similar tendency of association was observed ($P = 0.09$). This should be tested in larger samples.

Although the C allele of SNP5, a protective SNP, was associated with significantly lower mRNA levels of *HSP90B1*, the *HSP90B1* mRNA levels in lymphoblastoid cells did not show a significant difference between controls and patients. Although the possible effects of medication cannot be totally ruled out, such effect should be minimized because we cultured the lymphoblastoid cells at least for 1 month in culture media free from psychotropic drugs. It is not known how lower expression leves of *HSP90B1* reduce a risk for bipolar disorder in Japanese. HSP90B1, or GRP94, is an ER chaperone which binds to various proteins and peptides in a non-specific manner (Argon and Simen 1999). It also serves as a major Ca^{2+} buffer of the ER (Nigam et al. 1994). Overexpression of GRP94 is reported to protect the cells from death (Bando et al. 2004). Thus, the protective effect of the allele reducing the expression level of HSP90B1 against bipolar disorder is apparently contradictory to a hypotheseis that vulnerability to cell death may play a role in the pathophysiology of bipolar disorder (Manji and Duman 2001). However, it is not known whether gene expression in lymphoblastoid cells reflects the alteration in the brain. Transcriptional regulation may differ between lymphoblastoid cells and neuronal cells. Thus, it is too premature to assume that subjects carrying SNP5 have lower expression levels of HSP90B1 in neuronal cells.

In conclusion, we found an association of rs17034977 in *HSP90B1* with bipolar disorder in a Japanese population and suggested that the alteration of mRNA expression by this SNP could be an underlying mechanism for bipolar disorder.

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