Table 6 No association of LIM with schizophrenia in case control samples

Schizophrenia		*HWE*	*n*	*Allele*		*P-value**	*Genotype*			*P-value**	*Frequency*
				A	*G*		*A/A*	*A/G*	*G/G*		
SNP1	SC	0.5859	555	408	702		72	264	219		0.3676
rs10008257	CT	0.7233	562	424	700	0.6617	78	268	216	0.8847	0.3772
				A	G		A/A	A/G	G/G		
SNP2	SC	0.3868	562	179	945		17	145	400		0.1593
rs2433320	CT	0.1825	563	186	940	0.7316	11	164	388	0.2655	0.1652
				T	C		T/T	T/C	C/C		
SNP3	SC	0.8822	557	866	248		336	194	27		0.2226
rs2433327	CT	0.8525	565	875	255	0.8794	338	199	28	0.9890	0.2257
				T	C		T/T	T/C	C/C		
SNP4		0.3915	561	179	943		17	145	399		0.1595
rs2438146		0.1491	566	182	950	0.9542	10	162	394	0.2599	0.1608
				T	C		T/T	T/C	C/C		
SNP5	SC	0.7230	562	317	807		43	231	288		0.2820
rs2438140	CT	0.7967	568	338	798	0.4307	49	240	279	0.7210	0.2975
				A	G		A/A	A/G	G/G		
SNP6	SC	0.7311	558	348	768		56	236	266		0.3118
rs2452563	CT	0.4661	564	361	767	0.6830	54	253	257	0.6943	0.3200
				A	C		A/A	A/C	C/C		
SNP7	SC	0.6996	560	855	265		328	199	33		0.2366
rs2433324	CT	0.1083	567	856	278	0.6576	316	224	27	0.3234	0.2451
				T	G		T/T	T/G	G/G		
SNP8	SC	0.9901	557	679	435		207	265	85		0.3905
rs2452574	CT	0.2609	559	651	467	0.1958	196	259	104	0.3244	0.4177
				A	G		A/A	A/G	G/G		
SNP9	SC	0.8635	554	549	559		135	279	140		0.4955
rs2452578	CT	0.3382	554	583	525	0.1608	159	265	130	0.2610	0.5262
				A	G		A/A	A/G	G/G		
SNP10	SC	0.7339	560	459	661		96	267	197		0.4098
rs902981	CT	0.0419	562	470	654	0.6998	110	250	202	0.4567	0.4181
				A	G		A/A	A/G	G/G		
SNP11	SC	0.6983	562	660	464		196	268	98		0.4128
rs4634230	CT	0.0477	566	648	484	0.4950	197	254	115	0.4249	0.4276
				A	G		A/A	A/G	G/G		
SNP12	SC	0.5774	563	552	574		132	288	143		0.4902
rs12510147	CT	0.7924	568	519	617	0.1192	117	285	166	0.2882	0.4569
				A	G		A/A	A/G	G/G		
SNP13	SC	0.5110	555	655	455		197	261	97		0.4099
rs6854173	CT	0.0634	560	630	490	0.1986	188	254	118	0.3123	0.4375
				A	G		A/A	A/G	G/G		
SNP14	SC	0.8526	564	430	698		83	264	217		0.3812
rs12641023	CT	0.3903	568	457	679	0.3221	87	283	198	0.4470	0.4023
				T	C		T/T	T/C	C/C		
SNP15	SC	0.8366	559	418	700		77	264	218		0.3739
rs951613	CT	0.3439	565	452	678	0.2094	85	282	198	0.3833	0.4000
				A	G		A/A	A/G	G/G		
SNP16	SC	0.7090	561	437	685		83	271	207		0.3895
rs14082	CT	0.3502	567	470	664	0.2295	92	286	189	0.4413	0.4145

**P*-values are calculated by Fisher's exact test.

demonstrated, none of the patients homozygous for the SNP15 had lower *LIM/MLC1* ratio (Figure 3). This suggests that none of the patients tested had deletion of this region, and it is unlikely that there is copy number variation in this region.

Table 7 No association of *LIM* haplotype with schizophrenia in case–control samples

		2SNPs			3SNPs			
SNP1	rs10008257	0.8672			0.6616			
SNP2	rs2433320		0.9189			0.9900		
SNP3	rs2433327			0.9788			0.6108	
SNP4	rs2438146	0.7017						0.6275
SNP5	rs2438140		0.4507		0.5834			
SNP6	rs2452563			0.4941		0.3536		
SNP7	rs2433324	0.2144					0.3304	
SNP8	rs2452574		0.3313					0.3044
SNP9	rs2452578			0.1774	0.3593			
SNP10	rs902981	0.2320				0.1091		
SNP11	rs4634230		0.2279				0.2595	
SNP12	rs12510147			0.2499				0.4428
SNP13	rs6854173	0.4548			0.4579			
SNP14	rs12641023		0.2304			0.4337		
SNP15	rs951613			0.3947				
SNP16	rs14082							

Association studies in schizophrenia

Since original findings in DNA microarray analysis of the postmortem brains and lymphoblastoid cell lines suggested the association with schizophrenia, we further examined the association of *LIM* with schizophrenia (Table 6). There was no significant difference of allele and genotype frequencies between patients with schizophrenia and controls. Haplotype analysis of two or three SNPs also showed no significant association with schizophrenia. (Table 7).

In the family-based association study, none of the SNPs were significantly associated with schizophrenia. None of two or three SNPs haplotypes were associated with schizophrenia (Table 8).

Discussion

In this study, upregulation of *LIM* in the postmortem frontal cortex of patients with bipolar disorder and schizophrenia was confirmed in a different sample set. The upregulation could not be explained by confounding factors, such as medications, pH, and suicide status. These findings support the pathogenetic

Table 8 Family-based association analyses in schizophrenia

SNP ID		PDT SUM PDT	PDT AVE PDT	ETDT allele	TRANSMIT (Individual, Global, Common) 2SNPs					3SNPs			
SNP1	rs10008257	0.065	0.195	0.385	0.2178								
					0.6055					0.1992			
SNP2	rs2433320	0.695	0.682	0.416	0.6055	0.1967				0.6136	0.1967		
SNP3	rs2433327	0.225	0.365	0.249		0.3779	0.1967			0.6136	0.5796		
SNP4	rs2438146	0.692	0.677	0.407		0.3779	0.4085	0.3267			0.5796	0.2798	
SNP5	rs2438140	0.405	0.562	0.380			0.4085	0.5673	0.1564			0.7722	0.3167
SNP6	rs2452563	0.571	0.702	0.554	0.0966			0.5673	0.4552	0.0966		0.7722	0.7211
					0.2220				0.7133	0.2595			0.8331
SNP7	rs2433324	0.872	0.928	0.623	0.2220	0.1092				0.5829	0.0826		
						0.4107					0.2211		
SNP8	rs2452574	0.726	0.919	1.000		0.4107	0.1524				0.8382	0.1103	
							0.5583					0.4452	
SNP9	rs2452578	1.000	0.757	0.906			0.5583	0.1009				0.5361	0.0359
								0.3555		0.0460			0.2655
SNP10	rs902981	0.581	0.470	0.586				0.3555	0.1572	0.2298			0.3746
SNP11	rs4634230	0.355	0.288	0.333	0.0588				0.2340	0.2431	0.0588		
					0.1431				0.6585		0.1525	0.0833	
SNP12	rs12510147	0.682	0.726	1.000	0.1431	0.0484					0.2964	0.2439	
						0.1437	0.1153					0.2421	0.1015
SNP13	rs6854173	0.294	0.220	0.235		0.1437	0.4620			0.1064			0.2678
SNP14	rs12641023	0.493	0.181	0.231			0.4620	0.2852		0.4520	0.1025		0.4635
								0.3806	0.1045	0.4520	0.2290		
SNP15	rs951613	0.607	0.261	0.398				0.3806	0.1943		0.3650		
SNP16	rs14082	0.866	0.496	0.721					0.1943				

role of *LIM* in bipolar disorder and schizophrenia. Further, genetic association analyses suggested that polymorphisms of *LIM* confer a risk for bipolar disorder.

LIM, encoding a protein named enigma homolog (ENH), was initially identified by two hybrid screenings of rat brain cDNA library to interact with the regulatory domain of PKCβI. Northern blot analysis showed that there are two isoforms of *LIM*, 1.9 kb mRNA predominantly expressed in heart and skeletal muscles and 4.4 kb mRNA expressed in various tissues including the brain.[35] This gene was recently renamed as *PDLIM5*.

LIM is named after the LIM domain, composed of 50–60 amino acids that are involved in protein–protein interaction. Using an ENH-specific antibody, Maeno-Hikichi[13] found that ENH is expressed in various regions of the brain, most notably in the hippocampus, cortex, thalamus, hypothalamus, amygdala, and selected regions of the cerebellum. ENH was present in presynaptic nerve terminals, shown by colocalization with synapsin I. ENH coprecipitated with N-type, but not with P/Q-type calcium channels. ENH interacts with PKCε but not with α, $\beta 2$, and γ. Thus, ENH was regarded as an adaptor protein that forms the PKCε-*ENH*-N-type Ca^{2+} channel complex. ENH facilitated the PKC modulation of N-type Ca^{2+} channel, by interacting the α_{1B} subunit of calcium channel. They concluded that formation of a kinase–substrate complex by an adaptor protein, ENH, is the molecular basis of specificity and efficiency of cellular signaling.

Wang and Friedman[36] reported that cytosolic PKCε was reduced in the postmortem brain samples of patients with bipolar disorder. PKCε is a common target of mood stabilizers, lithium, and valproate.[37] Our group reported that calcium channel α_{1A} subunit gene was significantly downregulated in the postmortem brain samples of bipolar disorder patients. Calcium signaling linked with phosphoinositide pathway has been regarded as one of the important molecular cascades related to the pathophysiology of bipolar disorder.[15] These findings suggest that the impairment of molecular cascade from PKC to calcium channel, which controls intracellular calcium levels in neurons via G-protein-coupled receptors, may become a genetic risk factor of bipolar disorder.

While the function of ENH in the brain is well characterized, that in lymphocytes is still unknown. In addition, it is not known how *PDLIM5* is regulated in the brain and lymphocytes. Thus, it is difficult to interpret why *PDLIM5* mRNA expression was increased in the brain but decreased in the lymphoblastoid cells in bipolar disorder and schizophrenia. Considering the complex interaction of transcription factors in the regulation of gene expression depending on the tissue types, such findings in the opposite direction might have arisen from single nucleotide polymorphisms altering the binding of transcription machinery.

Recently, Arinami *et al* (personal communication) performed association analysis of *LIM* and schizophrenia and found that several SNPs were significantly associated with schizophrenia. Among the SNPs significantly associated with schizophrenia, rs2433320 was also examined in this study. No significant association of this SNP with schizophrenia was observed in this study. Considering that a larger number of case–control samples were tested in this study (570 schizophrenic patients and 570 controls, while only 278 patients and 462 controls were studied by Arinami *et al*) and that no association was found in family-based association analysis, it cannot be totally ruled out that their initial findings were false-positive results. However, when the data of these two studies were combined, AA genotype of rs2433320 was significantly associated with schizophrenia in the total sample (schizophrenia, 33/840 (3.9%), control, 19/1025 (1.9%), $P=0.02$, Fisher's exact probability test). Thus, it might be possible that this SNP confers a genetic risk for schizophrenia. Further studies are needed to draw a definite conclusion.

Since the SNPs associated with bipolar disorder were not the same in two independent case–control samples and the observed association in the MPS sample is marginal, the association observed in bipolar disorder should also be interpreted with caution.

Further replication studies in independent patient populations and using trio samples will be required to validate the apparent association between SNPs in the upstream region of *LIM* and bipolar disorder.

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Altered RNA editing of serotonin 2C receptor in a rat model of depression

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Abstract

Altered RNA editing of serotonin 2C receptor (*HTR2C*) has been suggested to be involved in the pathophysiology of major depression. Here we examined RNA editing status of *HTR2C* in the learned helplessness (LH) rats, one of well-established animal models of depression. LH rats showed the significantly increased RNA editing of site E, and tendency for increased RNA editing of other editing sites. Treatment with fluoxetine, a selective serotonin reuptake inhibitor, or imipramine, a tricyclic antidepressant, affected the RNA editing status of the LH rats. Although, these antidepressants differentially altered RNA editing status, they commonly reduced RNA editing efficiency of site E. We further revealed that altered RNA editing in the LH rats and by antidepressants was not explained by altered expression of RNA editing enzymes or their substrates (adenosine deaminases that act on RNA, *HTR2C*, and spliced form of *HTR2C*). These results suggest that alteration of RNA editing of *HTR2C* may play a role in the pathophysiology of depression and action of antidepressants.

Keywords: *HTR2C*; SSRI; TCA; *ADAR*; Psychiatric disorders; Learned helplessness

1. Introduction

Multiple lines of evidence suggest the involvement of serotonin 2C receptor (*HTR2C*) with mental disorders. Pharmacological studies revealed the involvement of *HTR2C* in locomotion, appetite, sexual behavior, and anxiety (Barnes and Sharp, 1999). *HTR2C*-deficient mice exhibited abnormal control of feeding behavior and enhanced seizure susceptibility (Tecott et al., 1995). One functional polymorphism of Cys23Ser (Okada et al., 2004) of *HTR2C* associated with depression and bipolar disorder (Gutierrez et al., 2001; Lerer et al., 2001; Oruc et al., 1997), and tardive dyskinesia in schizophrenia (Segman et al., 2000). In addition, down-regulation of *HTR2C* was found in postmortem brains of patients with bipolar disorder and schizophrenia (Castensson et al., 2003; Iwamoto et al., 2004).

In addition to variations in genomic sequence, post-transcriptional modifications of *HTR2C* also have some pathophysiological significance (Seeburg, 2002; Seeburg and Hartner, 2003; Sodhi and Sanders-Bush, 2004). Before translation, transcript of *HTR2C* undergoes adenosine-to-inosine (A-to-I) type RNA editing, by which specific adenosine residues are converted into inosine residues by adenosine deaminases that act on RNA (*ADAR*s) (Bass, 2002; Maas et al., 2003; Reenan, 2001). Since inosine is read as guanosine by translation machinery, this modification leads to amino acid substitution. To date, at least five adenosine residues (termed as sites A–E) in the second intracellular loop of *HTR2C* have been found to be edited (Burns et al., 1997). As a result, amino acid substitutions at three sites occur (I157 to V or M, N159 to S, D or G, and I161 to V), generating theoretically 24 isoforms. Importantly, different *HTR2C* isoforms exhibit considerably different G-protein coupling efficiency, and the combination of isoforms were regulated in a brain-region specific manner (Burns

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et al., 1997; Fitzgerald et al., 1999; Herrick-Davis et al., 1999; Niswender et al., 1999; Wang et al., 2000).

Altered RNA editing of *HTR2C* was reported in postmortem brains of patients with schizophrenia, depression, or those who committed suicide (Gurevich et al., 2002; Iwamoto and Kato, 2003; Niswender et al., 2001; Sodhi et al., 2001). Although these works suggested the role of RNA editing in mental disorders, the results remain inconsistent (Dracheva et al., 2003; Gurevich et al., 2002; Iwamoto and Kato, 2003; Niswender et al., 2001; Sodhi et al., 2001). This is possibly because of the difficulty in controlling the confounding factors, such as medical treatments and cause of death. To overcome this, results of postmortem studies needed to be compared with the finding in other models. In animal models, fluoxetine, a selective serotonin reuptake inhibitor (SSRI), altered RNA editing of *HTR2C* (Gurevich et al., 2002), while some drugs, such as cocaine or reserpine did not affect RNA editing (Iwamoto and Kato, 2002).

Although none of currently available animal models of depression can completely mimic human depressive disorder, learned helplessness (LH) is one of the most validated animal models of depression (Overmier and Seligman, 1967; Telner and Singhal, 1984). After pretreatment with repeated inescapable shocks, animals with LH exhibit decreased ability to escape unfavorable situations. Since this depressive phenotype can be ameliorated by antidepressants, the LH model has been widely used for studying the depression and the actions of antidepressants. Here we examined RNA editing of *HTR2C* and expression levels of related genes in the LH rats, and assessed the effect of antidepressants administration.

2. Materials and methods

2.1. Learned helplessness (LH) rats and drug administration

Detailed methods for creation of the LH rats were described elsewhere (Nakatani et al., 2004). The behavioral procedures were summarized in Fig. 1. Male Sprague-Dawley rats (5–6 week old) were purchased from Japan SLC (Shizuoka, Japan). Antidepressants were purchased from SIGMA (St. Louis, MO, USA). After 1 week of handling, they were used for experiments. On day 1, rats were subjected to inescapable foot electroshock pretreatment (0.5 mA, 10 s duration, shock interval 1–5 s, 160 trials) in a Plexiglas chamber. Control rats were placed for 1 h in the same chambers without electroshocks. On day 2, avoidance training was started in the same chamber, which was divided into two compartments using a partition. The partition included a gate, through which animals could move into the adjacent compartment. Rats were subjected to 15 avoidance trials with 30 s intervals. In each trial, 0.5 mA of current was applied via the grid floor during the first 3 s. If a rat moved to

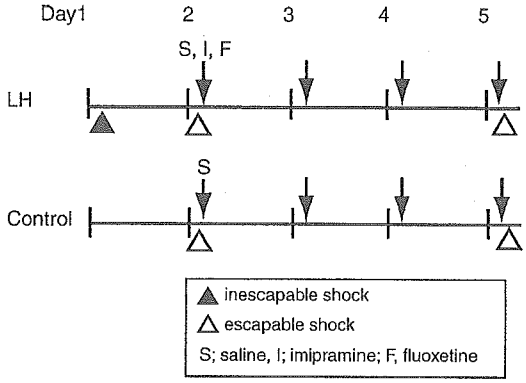

Fig. 1. Schematic representation of procedures. To make the LH rats, rats were first given inescapable shock on day 1 and then they were given escapable shock on day 2. The rats that showed greater than 50% failure in escape responses were selected as LH rats. LH rats were administered saline or antidepressants. On day 5, LH rats were given escapable shock to examine their LH status. Control rats were treated in the same way as the LH rats, but they were not given inescapable shock on day 1.

the other compartment within this period (escape response), the shock was terminated. Failures in escape response were counted as a measure of LH when they showed eight or more failures during a session. In this condition, LH was induced with a success rate of less than 40% (Nakatani et al., 2004). Control rats and subgroup of LH rats were administered saline (LH-S). The remaining LH animals were treated with either imipramine (25 mg/kg, i.p.) (LH-I) or fluoxetine (5 mg/kg, i.p.) (LH-F). Antidepressants or saline administration was administered once a day, starting in day 2 after the avoidance trial. On day 5, 30 min after the final administration, rats were tested for escape ability under escapable electroshock conditions. Antidepressants-treated rats that showed a 50% or more successful escape response were used for analysis. In our study, imipramine treatment recovered all rats ($n = 9$), and fluoxetine treatment recovered five out of seven from LH status (Nakatani et al., 2004). Rats were decapitated on day 6, 24 h after the final electroshock procedure. After the exclusion of olfactory bulb, coronal sections of prefrontal cortices (3 mm in length, right side) were cut and used in this study. Since altered RNA editing of *HTR2C* has been reported in the frontal cortex of patients with mental disorders, we chose and examined rat frontal cortex region in this study. Efforts were made not to be contaminated with striatum and choroids plexus in the samples.

2.2. Total RNA isolation and quantitative RT-PCR

Total RNA was extracted using an ISOGEN kit (NIPPON Gene, Toyama, Japan) and it was purified with an RNAeasy column (Qiagen, Valencia, CA, USA) as described by the manufacturer's protocol. One microgram of total RNA was used for cDNA synthesis by SuperScript II reverse transcriptase (Invitrogen, Carlsbad, CA, USA) and oligo(dT) primers (Invitrogen). Quantitative RT-PCR was

performed with an ABI PRISM 7900HT (Applied Biosystems, Foster city, CA, USA) using SYBER/GREEN I dye (Applied Biosystems). The comparative threshold cycle (C_t) method was employed for quantification of transcripts according to the manufacture's protocol (Applied Biosystems). Measurement of ΔC_t was performed at least three times per sample. Amplification of the single product was confirmed by monitoring the dissociation curve. We used two control genes (glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*), and β-actin (*ACTB*)) for normalization to control for possible fluctuations of the target transcripts. Primer pairs used in this study were as follows: *GAPDH*: 5′-GCCTGGAGAAACCTGCCAAGTAT-3′, and 5′-AGACAACCTGGTCCTCAGTGTAGC-3′; *ACTB*: 5′-ATCAAGATCATTGCTCCTCCTGAG-3′, and 5′-ACATCTGCTGGAAGGTGGACA-3′; *HTR2C*: 5′-TATCGCTGGACCGGTATGTAGC-3′, and 5′-GCAATCTTCATGATGGCCTTAGTC-3′; *HTR2C* (truncate form): 5′-GCCCCGTCTGGATTTCACTAGATGTGCTAT-3′, and 5′-AGGAACTGAAACTCCGGTCCAGCGATAT-3′; *ADAR1*: 5′-TTTCACCTCTACATCAGCACGGC-3′, and 5′-GCTTTCCACAGCACGGTCACT-3′; *ADAR2*: 5′-AGCGGATCTCCAACATAGAGGAC-3′, and 5′-TGCCTCTGCATTGCTGATACC-3′.

2.3. Cloning and sequencing analysis

Two rounds of RT-PCR amplification of rat *HTR2C* for estimation of RNA editing efficiency was performed as reported previously (Iwamoto and Kato, 2002). Primers used in the first round PCR were P1: 5′-TGGATTTCACTAGATGTGCT-3′, and P2: 5′-GTCCCTCAGTCCAATCACAG-3′. Those used in the second PCR were P1 and P3: 5′-TTGATATTGCCCAAACGATG-3′. cDNA synthesis and RT-PCR analysis was performed in each animal sample. A portion of product was cloned into the pCR2.1 vector (Invitrogen), and the reaction mixture was used for transformation of *E. coli*. cDNA clones derived from single bacterial colonies were sequenced with an ABI3700 DNA sequencer (Applied Biosystems).

3. Results

3.1. Altered RNA editing of HTR2C in the LH rats and by antidepressants

There are five RNA editing sites (sites A–E) at the 3′-end of exon 5 in *HTR2C* (Fig. 2A). RT-PCR products including these edited sites were used for cloning and sequencing analysis. To ensure reliable estimation of RNA editing efficiency, at least 50 colonies were sequenced per sample (control rats, $n = 4$; LH-S, $n = 4$; LH-I, $n = 4$; LH-F, $n = 5$). RNA editing efficiency at site E was significantly increased in the LH-S rats ($p = 0.034$) compared with control rats (Fig. 2B). We then assessed the effects of antidepressants, fluoxetine, and imipramine, on RNA editing of *HTR2C* in the LH rats. Administration of these drugs to LH rats significantly reversed the LH condition assessed by the escape test in our experimental system (Nakatani et al., 2004). Imipramine administration to LH (LH-I) rats showed a trend for decrease at E site ($p = 0.081$), compared with LH-S rats (Fig. 2B). Fluoxetine administration to LH (LH-F) rats decreased the RNA editing in the three editing sites (site A, $p = 0.069$; site B, $p = 0.080$; site E, $p = 0.002$) compared with LH-S rats (Fig. 2B).

Given the altered RNA editing among LH groups, we then compared the distribution of five major isoforms (see Fig. 2A for relationship between RNA editing and isoforms) to examine the functional consequence of altered RNA editing by antidepressants. Although, the composition of two major isoforms, VNV and VNI isoforms did not differ among the groups, a trend for increase of the VSV isoform in the LH-I rats ($p = 0.075$) and a significant decrease of the VSI isoform in the LH-F rats ($p = 0.032$) were found (Fig. 3). The non-edited INI isoform was decreased in the LH-I ($p = 0.098$) and LH-F ($p = 0.034$) rats compared with LH-S rats (Fig. 3).

3.2. Expression levels of HTR2C, splice variant of HTR2C (HTR2C-tr), ADAR1, and ADAR2 in the LH rats

To examine the possible cause of altered RNA editing of *HTR2C*, we examined expression levels of major genes involved in RNA editing (Yang et al., 2004) by quantitative RT-PCR. The *HTR2C* splice variant (*HTR2C*-tr) generated by alternative splicing within exon 5 does not contain any editing sites, and was suggested to be a non-functional form since translation was immaturely terminated in exon 6 (Canton et al., 1996; Xie et al., 1996) (Fig. 2A). It has been reported that there is a functional coupling between splicing and RNA editing events (Flomen et al., 2004; Raitskin et al., 2001). However, we did not find any alterations in the expression levels of these two forms (Fig. 4). The ratio of splice/non-splice variant was also not changed (Fig. 4).

In cellular models, global increase of *HTR2C* RNA editing in the human glioblastoma cells treated with interferon-α was associated with increased expression of *ADAR1* (Yang et al., 2004). Although significant change was seen only in site E, other editing sites also showed a tendency for increase in the LH rats. Thus, it is possible that these changes may be associated with altered expression of *ADAR1*. However, the expression levels of *ADAR1* as well as *ADAR2* were not significantly differed among groups (Fig. 5).

4. Discussion

Here, we showed the altered RNA editing of *HTR2C* in behavioral animal model of depression, learned helplessness. LH rats showed significant increase of RNA

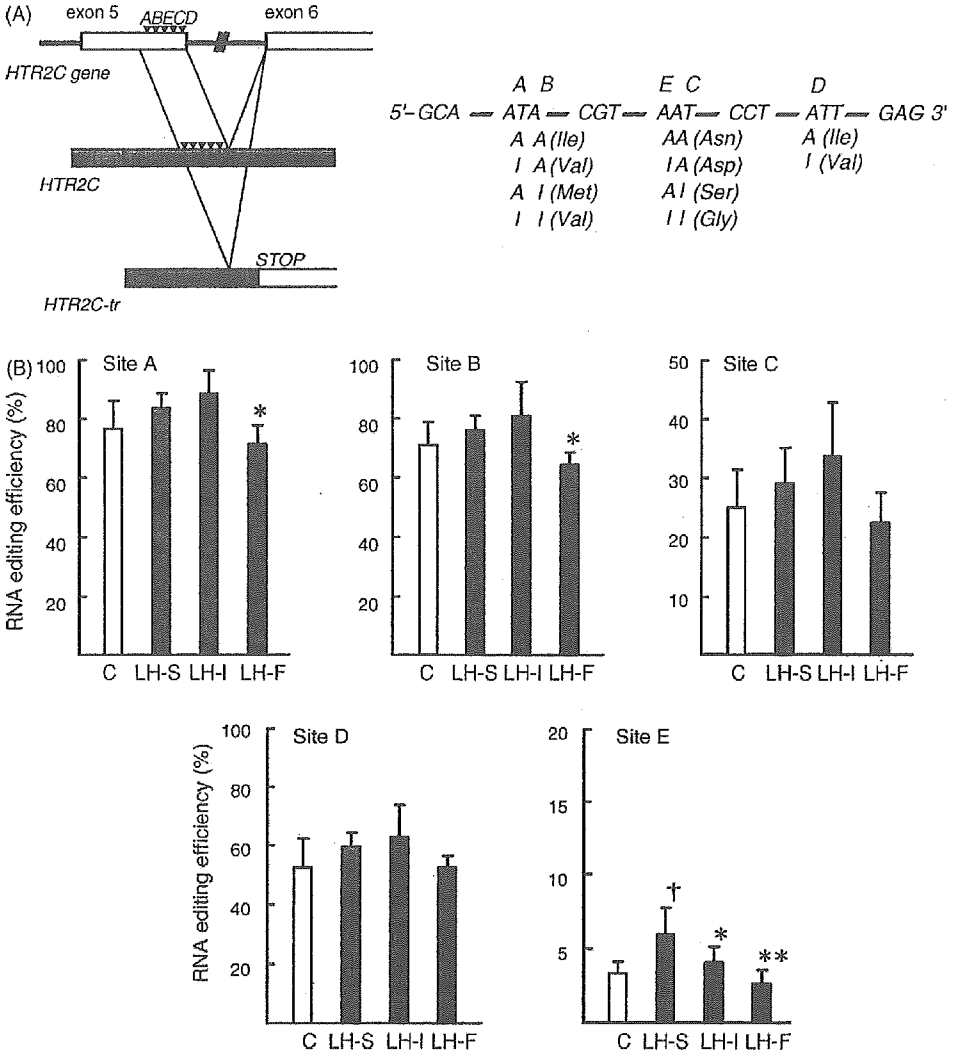

Fig. 2. RNA editing efficiencies of *HTR2C* in the LH rats. (A) Genomic context and transcripts of *HTR2C* around RNA editing sites (*left*), and amino acid changes associated with RNA editing of the *HTR2C* (*right*). (B) RNA editing efficiency of each site (mean ± S.D.). *HTR2C* cDNA derived from single bacterial colonies were sequenced in at least 50 clones per sample (control rats, $n = 4$; LH-S, $n = 4$; LH-I, $n = 4$; LH-F, $n = 5$). The sum of the number of sequenced clones that contained the *HTR2C* was 1.114. Data were compared by one-way ANOVA ($p < 0.05$), and statistical analysis were then conducted post hoc using Dunnett's test with LH-S as a reference group. $^{\dagger}p < 0.05$ in control rats vs. LH-S rats. $^{*}p < 0.10$, $^{**}p < 0.05$ in LH-S rats vs. either LH-I or LH-F rats.

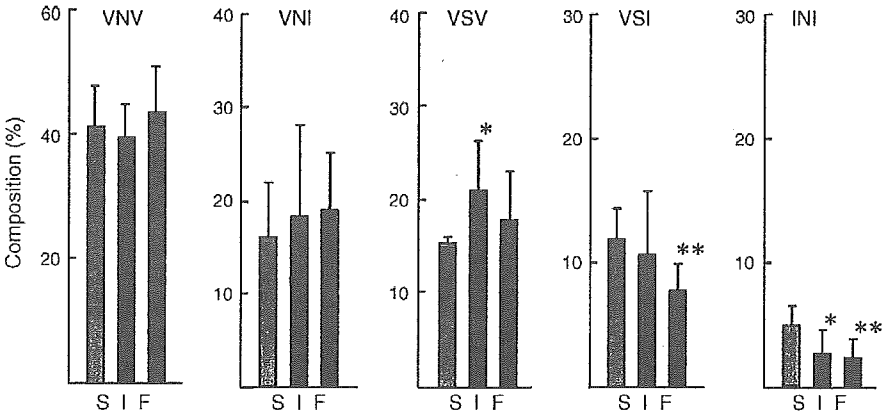

Fig. 3. Comparison of the composition of the major *HTR2C* isoforms. Data (mean ± S.D.) were compared by *t*-test. $^{*}p < 0.10$, $^{**}p < 0.05$ in LH-S rats vs. either LH-I or LH-F rats.

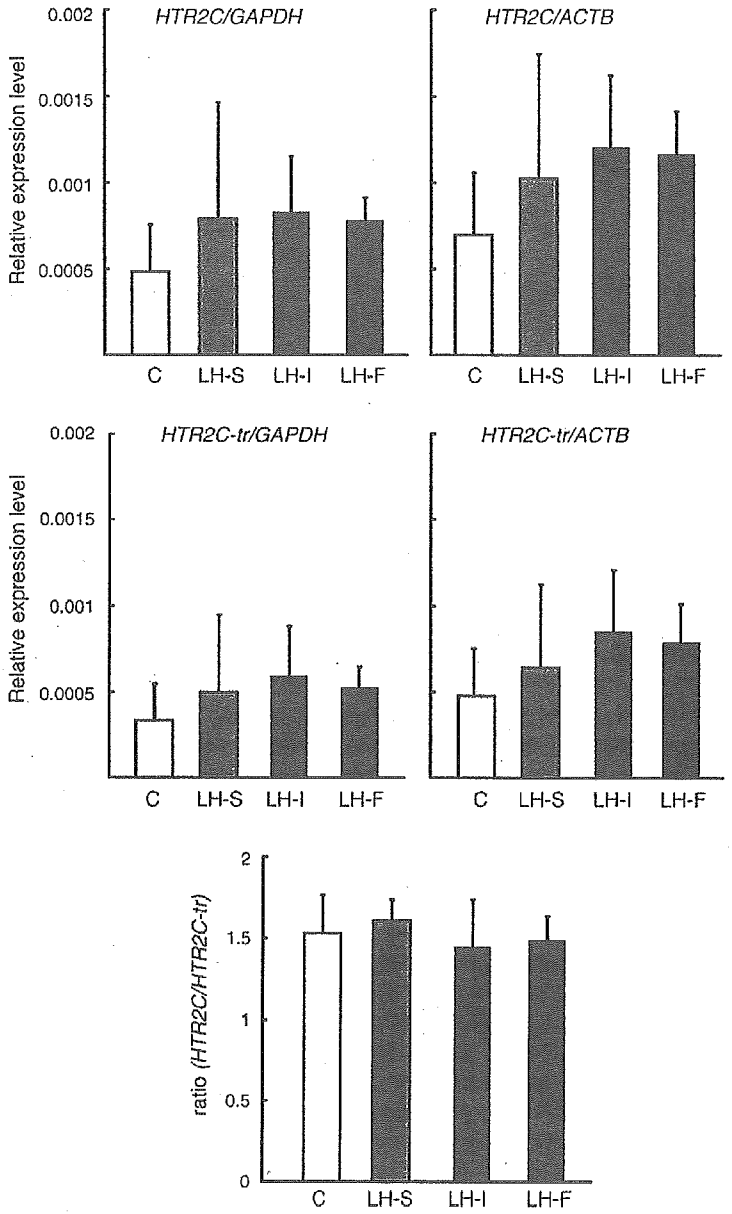

Fig. 4. Expression levels of *HTR2C* and *HTR2C*-tr. Data (mean ± S.D.) were compared by *t*-test. There were no statistical differences ($p < 0.10$) among groups. Control rats, $n = 4$; LH-S, $n = 4$; LH-I, $n = 4$; LH-F, $n = 5$.

editing of site E. Two different classes of antidepressants, imipramine and fluoxetine, reduced the RNA editing of site E in LH rats. These results suggest that altered RNA editing of *HTR2C*, especially RNA editing of site E, is associated with depressive state in this animal model.

The limitations of our findings were the specificity of altered RNA editing in LH rats and by the antidepressants. In this study, we did not analyze RNA editing status in the rats that failed to show LH status, and the LH rats that failed to recover from LH status by antidepressant treatments. However, considering the laborious work of conventional cloning and sequencing analysis, it would not be a practical approach to examine the various experimental groups. We have previously reported the method for estimating RNA editing efficiency by primer extension combined with denaturing high-performance liquid chromatography (Iwamoto and Kato, 2002). Although this method was rapid and accurate, there is a disadvantage in that this can only estimate the RNA editing efficiency of site A or D, which located at either ends of RNA editing region. Therefore, other high throughput method should be developed to estimate the RNA editing efficiency of other editing sites. In addition, given the high recovery rate of antidepressants treatment (100% in imipramine, and 71% in fluoxetine), we could not assess the RNA editing status of the LH rats that failed to recover from LH status by antidepressants.

It has been reported that fluoxetine administration in normal mice (129Sv) showed significantly decreased RNA

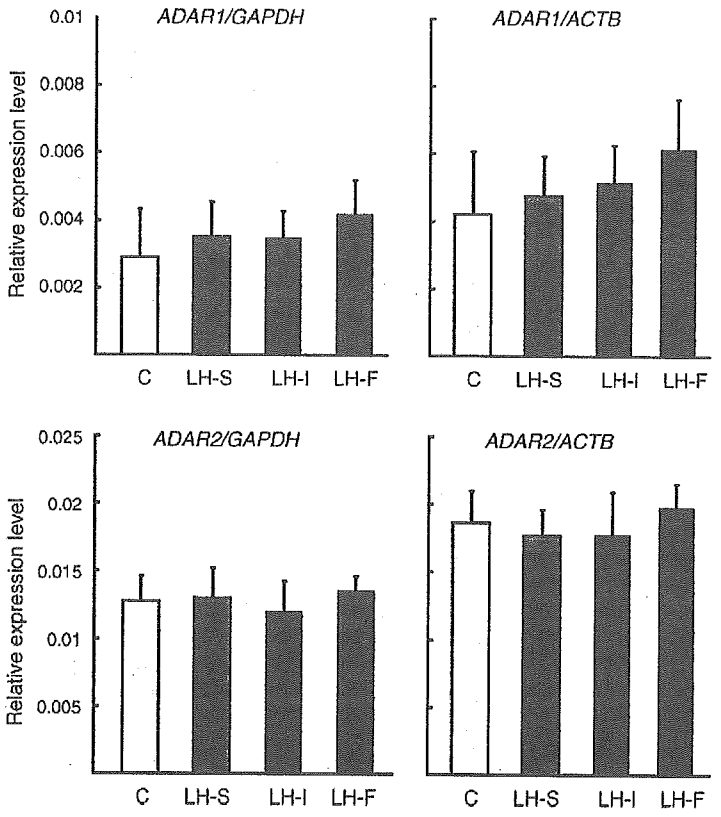

Fig. 5. Expression levels of *ADAR1* and *ADAR2*. Data (mean ± S.D.) were compared by *t*-test. There were no statistical differences ($p < 0.10$) among groups. Control rats, $n = 4$; LH-S, $n = 4$; LH-I, $n = 4$; LH-F, $n = 5$.

editing in site E, decrease in site C, and increased RNA editing in site D (Gurevich et al., 2002). Our findings confirmed the effect of fluoxetine on RNA editing with regard to site E. Although we also showed that fluoxetine reduced RNA editing efficiency at other sites, this may not be relevant to antidepressants efficacy, because imipramine had an opposite effect on RNA editing except site E. Tricyclic antidepressants, such as imipramine inhibit the reuptake of both serotonin and norepinephrine by acting on monoamine transporters, whereas SSRI, including fluoxetine specifically block reuptake of serotonin. These differences in pharmacological profiles may account for the differential effect on RNA editing efficiency.

At the isoform level, both fluoxetine and imipramine decreased the fully non-edited, INI isoform (Fig. 3). Considering that the INI isoform exhibits the greatest basal activity, agonist affinity and potency (Herrick-Davis et al., 1999), decreased levels of this isoform in LH rats treated with antidepressants may result in the reduction of sensitivity of *HTR2C* receptor functions. Although other changes at the isoform level are not common to two antidepressants in this study, they may also reduce the sensitivity of *HTR2C*, since the VSI isoform exhibit relatively high sensitivity among the *HTR2C* isoforms, whereas the VSV isoform exhibits low sensitivity (Herrick-Davis et al., 1999).

Recently, similar results have been reported in mice that were subjected to a modified forced swimming test (FST) (Englander et al., 2005). Mice (Balb/c) subjected to the FST showed global increased RNA editing of *HTR2C*, and chronic fluoxetine-treated mice exposed to the FST reversed this increased RNA-editing (Englander et al., 2005). Although alteration at the isoform level, such as content of the INI isoform, was not inconsistent with our results, this may be partly attributable to the species-specific variation. Interestingly, there is a great variation in the distribution of *HTR2C* isoforms, especially in content of the INI isoform, among different inbred strains of mice (Englander et al., 2005). Furthermore, effect of chronic fluoxetine on the content of the INI isoform was opposite between C57Bl/6 (increased) and Balb/c (decreased) (Englander et al., 2005). In addition, alterations at the isoform level were needed to be assessed with caution since functional properties of all isoforms have not been fully determined. Therefore, it may be possible that presence or absence of minor isoforms may have some significance of the total *HTR2C* receptor function.

We revealed that the expression levels of major genes involved in RNA editing of *HTR2C* were not changed. Therefore, molecular mechanism of the altered RNA editing of *HTR2C* in the LH rats and by antidepressants remains to be explored. There would be several other possible

explanations, such as the expression level of HBII-52 small nucleolar RNA, which has sequence complementary to the mRNA containing the *HTR2C* editing sites (Cavaille et al., 2000), and cellular localization of *ADAR*s (Desterro et al., 2003; Poulsen et al., 2001; Sansam et al., 2003; Yang et al., 2003). Therefore, further examination of such possibilities would be helpful for understanding the altered RNA editing of *HTR2C* in this animal model.

Acknowledgments

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Functional polymorphisms of HSPA5: Possible association with bipolar disorder

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Abstract

Altered endoplasmic reticulum stress (ER) response signaling is suggested in bipolar disorder. Previously, we preliminarily reported the genetic association of *HSPA5* (*GRP78/BiP*) with bipolar disorder. Here, we extended our analysis by increasing the number of Japanese case-control samples and NIMH Genetics Initiative bipolar trio samples (NIMH trios), and also analyzed schizophrenia samples. In Japanese, nominally significant association of one haplotype was observed in extended samples of bipolar disorder but not in schizophrenia. In NIMH trios, no association was found in total samples. However, an exploratory analysis suggested that the other haplotype was significantly over-transmitted to probands only from the paternal side. The associated haplotype in Japanese or NIMH pedigrees shared three common polymorphisms in the promotor, which was found to alter promotor activity. These findings suggested promotor polymorphisms of HSPA5 may affect the interindividual variability of ER stress response and may confer a genetic risk factor for bipolar disorder.

Keywords: Bipolar disorder; Schizophrenia; Endoplasmic reticulum stress; HSPA5/GRP78; Association study; Promotor assay

Bipolar disorder is a severe mental disorder characterized by recurrent episodes of mania and depression, affecting about 0.5–1% of the population [1]. Although the contribution of genetic factors has been evidenced by family, twin and adoption studies, the molecular pathophysiology of the illness has been controversial [2,3]. Recently, we suggested that the endoplasmic reticulum (ER) stress response signaling is one of candidate cascades related to pathology of the illness [4].

In our previous study, *XBP1* and *HSPA5* were down-regulated in the lymphoblastoid cells of monozygotic twins with bipolar disorder compared with healthy co-twins by DNA microarray analysis. Induction of *XBP1* and *HSPA5* mRNA by thapsigargin was reduced in the patients' cell lines and valproate induced *ATF6* mRNA expression and enhanced the ER stress response in SHSY5Y cells [4]. Although we also reported that a functional polymorphism of *XBP1* (−116C/G) altering the ER stress response was

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associated with bipolar disorder, the genetic association was not replicated in Caucasian bipolar samples and Taiwanese samples [5,6]. On the other hand, the association of *XBP1* −116C/G polymorphisms with schizophrenia was observed in Chinese samples and Japanese samples [7,8]. Schizophrenia is another major mental disorder sharing common clinical features and genetic background with bipolar disorder [9]. The chromosomal region of *XBP1*, 22q, is one of common linkage loci for these two disorders. Thus, altered ER stress response signaling may contribute to the pathophysiology of both of these major mental disorders.

When unfolded proteins accumulate in endoplasmic reticulum (ER) by some reasons, ER stress response begins. ER stress response consists of four signaling cascades: (1) induction of ER chaperon such as *HSPA5* (*GRP78/BiP*), which promotes the folding of unfolded proteins (unfolded protein response, UPR), (2) inhibition of protein synthesis, (3) induction of ER-associated degradation pathway, which promotes the processing of unfolded proteins, and (4) induction of apoptosis when this system could not process the unfolded proteins [10,11].

In the previous paper, we focused on *XBP1* since it is a transcription factor regulating the mRNA expression of ER chaperon genes such as *HSPA5*. However, initial reaction eliciting ER stress response is the consumption of HSPA5. When HSPA5 proteins are consumed to fold unfolded proteins, dissociation of HSPA5 from ATF6 protein on the ER membrane causes cleavage of ATF6. Cleaved ATF6 protein induces the expression of ER chaperons 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 active form XBP1 that strongly induces the expression of chaperon genes including *HSPA5* as well as *XBP1* itself [10]. In this regard, HSPA5 is a key protein regulating ER stress response.

HSPA5, *TRA1* (*GRP94*), and *CALR* (*Calreticulin*) are known to be increased in the temporal cortex of depressed subjects who died by suicide [12]. Anti-malarial drug mefloquine, which is known to cause psychiatric symptoms including bipolar disorder in susceptible individuals [13], is reported to induce ER chaperons including HSPA5 in rat neurons [14]. Methamphetamine (MAP), a psychostimulant causing manic state, is also known to induce *HSPA5* and other ER chaperon genes in the mouse brain [15]. Induction of *HSPA5* by mefloquine or MAP is interpreted that these drugs cause ER stress, since they also induce ER stress pathway other than UPR, such as apoptosis. On the other hand, valproate, one of the mood stabilizers, is known to increase *HSPA5* expression and have neuroprotective effects by enhancing the UPR [16–20,4]. Recently, the other mood stabilizer, lithium, was also shown to protect the rat PC12 cells against ER stress by inducing the *HSPA5* mRNA [21].

HSPA5 gene is located on 9q33–34.1, on which significant evidence for linkage with bipolar disorder was observed by several studies [22–24].

In this study, we examined whether or not genetic variations of *HSPA5* contribute to the pathophysiology of bipolar disorder and schizophrenia.

Part of the data presented in this paper (data on 3 of 6 SNPs in 195 of 439 patients with bipolar disorder and 254 of 492 controls in case-control studies, and 88 of 240 trios in transmission disequilibrium test) was reported in the reply to correspondence [5].

Materials and methods

Subjects. For the case-control study, 439 patients with bipolar disorder (50.5 ± 13.4 years old, 208 males and 231 females), 229 patients with schizophrenia (46.0 ± 14.9 years old, 131 males and 98 females), and 492 controls (41.7 ± 14.4 years old, 246 males and 246 females) were analyzed. In addition to the samples previously reported in the reply to correspondence [5], we increased the number of case-control samples including the independently collected sample set described previously as "MPS samples" for replication study [25]. MPS samples include 239 patients with bipolar disorder (51.0 ± 13.1 years old, 131 males and 108 females) and 234 controls (51.6 ± 10.7 years old, 117 males and 117 females). They were diagnosed according to the DSM-IV criteria (American Psychiatric Association). Controls were selected from students, nurses, office workers, and doctors in participating institutes, and their friends. A senior psychiatrist interviewed them and they did not have major mental disorders. Only a part of them were interviewed using a structured interview, Mini-International Neuropsychiatric Interview (M.I.N.I.) [26]. In Japanese, no significant population stratification has repeatedly reported in several studies including a part of our samples [4,27–29]. For transmission disequilibrium test, we analyzed total 240 trio samples (227 trios with BPI proband and 12 trios with BPII proband) from NIMH Genetics Initiative Pedigrees (http://zork.wustl.edu/nimh/), including 88 trios previously reported in the reply to correspondence [5]. Only one trio was obtained from one family. The criteria, by which the trio was selected from a pedigree, were, (1) DNA is available for parents and the proband, (2) if multiple complete trios were found in one pedigree, the trio with younger generation was selected, (3) if multiple trios were available in one generation, 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. Written informed consent was obtained from all the subjects. Postmortem brains were donated by The Stanley Medical Research Institute's Brain Collection courtesy of Drs. Michael B. Knable, E. Fuller Torrey, Maree J. Webster, Serge Weis, and Robert H. Yolken. The Ethics Committees of the Brain Science Institute (RIKEN) and participating institutes approved the study.

Mutation screening of the HSPA5 genes and genotyping of flag SNPs. Polymorphisms of all exons and the upstream region (1 kb) of *HSPA5* (GenBank Accession No. NT_008470) were screened by sequencing in 24 patients with bipolar disorder and eight patients with schizophrenia. Primer sequences are available on request. Genotyping was performed using commercially available TaqMan probes and ABI7900HT according to the protocol recommended by the manufacturer (Applied Biosystems, Foster City, CA, USA).

cDNA synthesis. To genotype the 3′-UTR of *HSPA5* mRNA, we generated cDNA. RNA samples were extracted from the postmortem brain tissues and single-strand cDNA was synthesized by the same method described previously [30].

Cell culture. We cultured SHSY5Y cells in the medium of DMEM (Sigma, Saint Louis, Missouri) containing 10% fetal bovine serum (FBS) and used for degradation assay and promotor assay.

Degradation assay. We generated constructs for mRNA degradation assay. We amplified a 3778 bp fragments excluding SV40 polyA signal region (1242 to 680) of pCMV-tag3 vector (Stratagene, La Jolla, CA, USA), a 1653 bp fragment (coding sequence of luciferase) of pGL3-basic vector (Promega, Madison, WI, USA), and a 1741 bp fragment of 3′-UTR of HSPA5 with SNP5 T. Using BD In-Fusion Technique (BD Biosciences Clontech, San Jose, CA, USA). We fused the three PCR fragments and generated a 7152 bp construct having CMV promotor, coding sequence of luciferase, and 3′-UTR of *HSPA5*. In this construct, start codon of pCMV-tag3 vector fell on that of luciferase coding sequence and the stop codon of luciferase coding sequence fell on the stop codon of the HSPA5 gene. Next we made mutation in SNP5 from T to C using QuickChange Site-Directed Mutagenesis Kit (Stratagene) and generated point mutated construct only in SNP5. The sequence of coding region and 3′-UTR was confirmed by sequencing. We transfected SHSY5Y cells cultured in a 96-well plate using Superfect (Qiagen, Valencia, CA, USA) with 0.2 μg of the reporter plasmid (SNP5 T or C) and 0.05 μg of a reference plasmid (pRL-CMV). After a 48-h incubation, with pre-incubation by the medium containing 5 μg/ml actinomycin D (Wako, Osaka, Japan) for 0, 2, 4, and 6 h before luciferase assay, we measured luciferase activities with the aid of Dual-Glo Luciferase assay system (Promega). In this assay, mRNA degradation was compared by the degradation of mRNA of generated vector and mRNA of pRL-CMV vector. By incubation with actinomycin D for 6 h, the activity of reporter or reference vector decreased to the approximately one-third level compared with the activity not-treated with actinomycin D. The assay was performed independently four times.

Promotor assay. We amplified a 548-bp fragment (−554 to −7, the numbers indicate the nucleotide positions from the transcription start site) of the *HSPA5* gene by PCR and cloned into the *MluI*/*BglII* site of pGL3-Basic vector (Promega). As a template, genomic DNA derived from control having heterozygotes of haplotype 1 and haplotype 3, or haplotype 2 and haplotype 4, was used and we prepared four kinds of reporter plasmids, having the sequence corresponding to haplotype 1, haplotype 2, haplotype 3 or haplotype 4. We also cloned the spliced *XBP1* cDNA into the *Bam*HI/*Hin*dIII site of pcDNA3.1 vector (Invitrogen, San Diego, CA, USA) to construct a spliced *XBP1*-expressing vector. For the experiments of thapsigargin treatment, we transfected SHSY5Y cells cultured in a 96-well plate with 0.2 μg of the reporter plasmid or pGL3-Basic vector carrying no insert and 0.1 μg of a reference plasmid (pRL-CMV) using Superfect (Qiagen). For the experiments to examine the effects of XBP1, we transfected SHSY5Y cells with 0.4 μg DNA containing 0.2 μg of the reporter plasmid, 0.2 μg pcDNA3.1 vector with or without the insert of spliced *XBP1*, and 0.1 μg of a reference plasmid (pRL-CMV). After a 48-h incubation, thapsigargin (300 nM) or vehicle was added. Three hours after the thapsigargin stimulation, we measured the luciferase activities with the aid of Dual-Glo Luciferase assay system (Promega). The assay was performed independently four times.

Data analysis. Linkage disequilibrium (LD) patterns were assessed by the standardized disequilibrium coefficient (D') and squared correlation coefficient (r^2) calculated by COCAPHASE and TDTPHASE programs (http://www.rfcgr.mrc.ac.uk/fdudbrid/software/unphased/). Analysis of haplotypic distribution, haplotype frequencies, and haplotype TDT analysis was performed using COCAPHASE and TDTPHASE programs. Global significance was calculated by random permutation test for 10,000 times using COCAPHASE and TDTPHASE programs. Two-sample t test was used for comparison among the haplotypes in the promotor assay and mRNA degradation assay.

Results

Mutation screening and identification of the haplotype block of HSPA5

We first performed mutation search of *HSPA5* gene by screening all exons and the upstream region (1 kb) in 24 patients with bipolar disorder and eight patients with schizophrenia. Four single nucleotide polymorphisms (SNPs) [rs391957 (A/G SNP1), rs17840762 (C/T SNP2), rs17840761 (C/T SNP3), and rs3216733 (G/deletion SNP4)] in the upstream region and 3 SNPs [rs16927997 (T/C SNP5), rs1140763 (C/T SNP6), and rs12009 (C/T SNP7)] in the 3′-untranslated region were identified (Fig. 1A). In these 32 samples, SNP1 and SNP4, SNP3 and SNP6 were completely linked. None of two non-synonymous (rs11542738 and rs11542739) and one synonymous

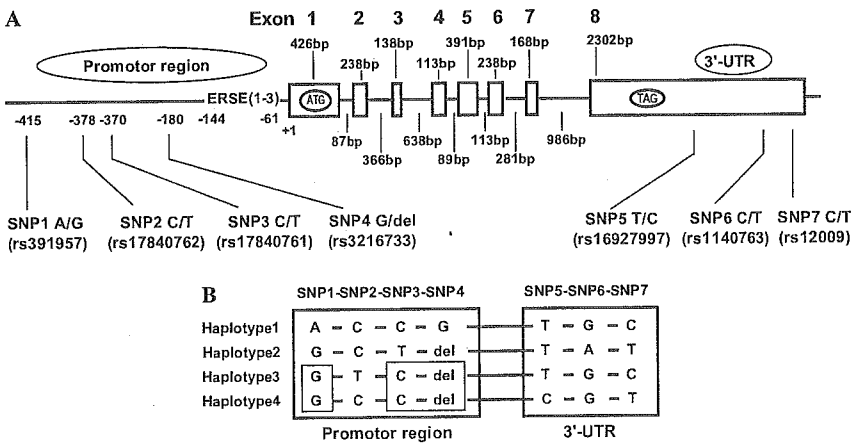

Fig. 1. *HSPA5* gene structure. (A) Genomic structure and the location of single nucleotide polymorphisms. (B) Haplotypes of 7 SNPs of *HSPA5*. Three SNPs in the upstream are common to haplotype 3, the risk for NIMH trios when transmitted paternally, and haplotype 4, the risk for Japanese population.

polymorphisms (rs11542736) registered in dbSNP database were detected in these patients. We genotyped six SNPs, except for SNP6 in 457 samples (257 control samples and 200 bipolar samples), and analyzed by COCAPHASE program. We confirmed that these six SNPs were in linkage disequilibrium (Table 1). We performed a genetic association study for four haplotypes consisted of these SNPs (haplotype 1; A-C-C-G-T-G-C, haplotype 2; G-C-T-del-T-A-T, haplotype 3; G-T-C-del-T-G-C, and haplotype 4; G-C-C-del-C-G-T) (Fig. 1B).

Case-control study of Japanese bipolar disorder and schizophrenia samples

By genotyping three flag SNPs (SNP1, SNP3, and SNP5), we performed association studies in Japanese case-control samples (control $n = 492$, bipolar disorder $n = 439$, and schizophrenia $n = 229$). Haplotype 4 was significantly associated with bipolar disorder ($p = 0.0489$) (Table 2), but not with schizophrenia. Information of family history was available in only a part of bipolar samples. As per our preliminary report [5], the association was much stronger in patients with family history of affective disorders including bipolar disorder and depression in their first-degree relatives ($p = 0.00099$). Extended samples included independently collected sample set, "MPS sample" as described in Materials and methods. In this sample set, no significant association of any haplotype was observed (data not shown).

TDT analysis in NIMH trios

We performed transmission disequilibrium test (TDT) in 240 parents and proband trios of bipolar disorder obtained from NIMH Genetics Initiative pedigrees (NIMH trios). In NIMH trios, mainly originated from Caucasians, only three haplotypes were identified. Haplotype 4 was not found and SNP5 was not polymorphic in NIMH samples. By haplotype TDT using TDTPHASE program, no significant over-transmission was observed (Table 3).

Exploratory analysis of sex-specific transmission

Next, TDT was also performed separately in paternal and maternal transmission, because the parent of origin

Table 1
Linkage disequilibrium (LD) patterns in this region

Control	D'					
	SNP1	SNP2	SNP3	SNP4	SNP5	SNP7
r^2						
SNP1		1	1	1	1	1
SNP2	0.07541		1	1	1	1
SNP3	0.4547	0.1026		1	1	1
SNP4	1	0.07541	0.4547		1	1
SNP5	0.04775	0.01065	0.06494	0.04775		1
SNP7	0.6222	0.1402	0.7317	0.6222	0.07596	
Case	D'					
	SNP1	SNP2	SNP3	SNP4	SNP5	SNP7
r^2						
SNP1		1	1	1	1	1
SNP2	0.07608		1	1	1	1
SNP3	0.3462	0.1061		1	1	1
SNP4	1	0.07608	0.3462		1	1
SNP5	0.07116	0.02182	0.09927	0.07116		
SNP7	0.5706	0.1775	0.598	0.5706	0.1229	

D' and r^2 were calculated by COCAPHASE.

Table 2
Association of HSPA5 haplotype with bipolar disorder in Japanese case-control samples

	BP				Sch		Control
	Total	p value	With FH[a]	p value		p value	
Haplotype 1	282(0.32)	0.0902	40(0.29)	0.0868	145(0.32)	0.1625	352(0.36)
Haplotype 2	373(0.42)	0.831	54(0.38)	0.322	204(0.45)	0.444	422(0.43)
Haplotype 3	116(0.13)	0.362	19(0.14)	0.555	49(0.11)	0.5903	116(0.12)
Haplotype 4	107(0.12)	0.0498*	27(0.19)	0.00099*	54(0.12)	0.1385	92(0.093)
	(Global 0.16)		(Global 0.0028)*		(Global 0.32)		

p values are calculated by COCAPHASE for only haplotypes which are certain at least once. BP, bipolar disorder; Sch, schizophrenia; FH, family history of mood disorders.

[a] The patients' population is the same as that reported in [5], in which SNP3, 4, and 7 were genotyped.

* $p < 0.05$.

Table 3
Transmission disequilibrium test of HSPA5 haplotype in NIMH trios

	Total			Maternal transmission			Paternal transmission		
	T	NT	p value	T	NT	p value	T	NT	p value
Haplotype 1	199(0.41)	195(0.40)	0.792	81(0.41)	77(0.39)	0.681	82(0.41)	84(0.42)	0.838
Haplotype 2	243(0.51)	254(0.53)	0.476	108(0.55)	107(0.54)	0.919	97(0.49)	108(0.55)	0.268
Haplotype 3	36(0.075)	29(0.060)	0.368	9(0.045)	14(0.070)	0.281	19(0.096)	6(0.030)	0.00597*
Haplotype 4	—	—	—	—	—	—	—	—	—
	(Global 0.59)			(Global 0.43)			(Global 0.0054)*		

p values are calculated by TDTPHASE. T, transmitted; NT, not-transmitted.
* $p < 0.05$.

effect has been shown in bipolar disorder [2]. This should be considered as an exploratory and hypothesis-generating analysis. In this study, significant over-transmission of haplotype 3 was found only in paternally transmitted alleles (transmitted, 19; not transmitted, 6; $p = 0.0059$) (Table 3).

Nominally significantly associated haplotype was observed as haplotype 4 in Japanese and haplotype 3 in NIMH trios. Three SNPs in the upstream region are common to these two haplotypes (Fig. 1B). When the association was re-analyzed using the haplotype of three SNPs (SNP1, 3, and 4) in the upstream region, significant association was observed in Japanese samples ($p = 0.031$ in total samples and $p = 0.0029$ in the samples with family history of mood disorder).

We hypothesized two possibilities. One is the contribution of SNP5 in Japanese samples. SNP5 is located in the 3′-UTR. Around the SNP, there are four ATTTA motifs with AT-rich contents (Fig. 2A), which suggested that the region is so-called "AU-rich element." AU-rich element is associated with mRNA stability, in most cases it promotes degradation of mRNA [31]. SNP5 is located next to the ATTTA motif and substitution from T to C reduces the contents of AT, which made us hypothesize that the substitution was associated with the stability of *HSPA5* mRNA. To test this hypothesis, we performed mRNA degradation assay. The other hypothesis is the contribution of haplotype in the upstream region, which was common to Japanese samples and NIMH trios. To test this hypothesis, we performed a promotor assay.

Degradation assay

We performed mRNA degradation assay using luciferase assay system. We generated two kinds of constructs which commonly have a CMV promotor and firefly luciferase coding sequence, with the 3′-UTR of *HSPA5*, either with T or C at SNP5. SHSY5Y cells were transfected by the construct and the degradation was examined by the relative activity of firefly luciferase of the reporter plasmid to that of *Renilla* luciferase of a reference plasmid co-transfected. We treated the transfected cells for 0, 2, 4, and 6 h with actinomycin D (5 μg/ml), which inhibits the transcription of mRNA, and the relative rate of degradation was calculated. No difference was observed between the construct with SNP5-T and the construct with SNP5-C (Fig. 2B).

Promotor assay

We cloned the promotor region of each haplotype into the pGL3-basic vector and generated four kinds of con-

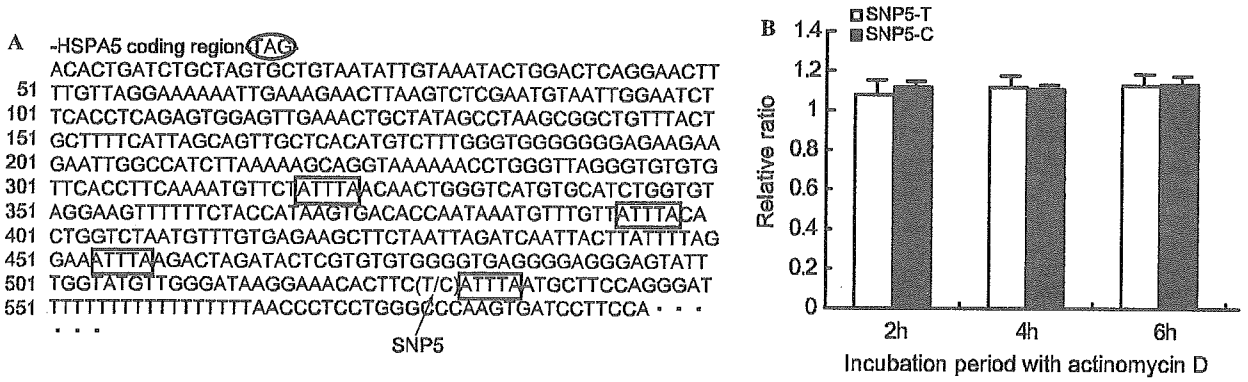

Fig. 2. Degradation assay. (A) 3′-UTR of *HSPA5* gene. There are four ATTTA motifs and SNP5 is located next to the fourth motif. (B) Degradation assay for the examination whether SNP5 is associated with mRNA degradation. No difference between SNP5-T construct and SNP5-C construct. *X*-axis indicates the incubation time with actinomycin D (5 μg/ml) and *Y*-axis indicates the relative ratio of relative activities compared with the activities at 0 h. Values are means ± SD. Assay was performed independently four times.

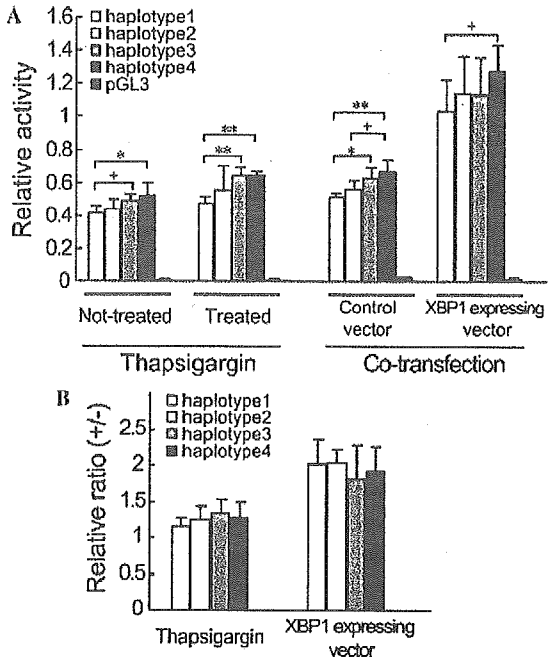

Fig. 3. Effects of haplotype for promotor activity. (A) Basal activity and the activity treated with 300 nM thapsigargin of the construct having haplotype 3 or haplotype 4 were significantly higher than that of haplotype 1, or show a tendency higher than that of haplotype 2. *Y*-axis is relative activity of firefly luciferase of reporter plasmid to *Renilla* luciferase of reference plasmid. Values are means ± SD. Asterisk indicates $p < 0.05$, double asterisk, $p < 0.01$ and plus sign $0.05 < p < 0.1$ (Two-sample *t* test)..Assay was performed independently four times. (B) The response to ER stress or XBP1 co-transfection. There was no difference among four haplotypes in the response rate. Vertical axis indicates the relative ratio of the activity treated with thapsigargin to the activity not treated, or the ratio of the activity in the cells co-transfected with spliced form XBP1-expressing vector to that in the cells with control vector. Values are means ± SD.

structs. SHSY5Y cells were transfected by the constructs with pRL-CMV vectors and the relative activity of firefly luciferase (pGL3) to *Renilla* luciferase (pRL) was examined. Among four constructs, the activity of constructs having haplotype 3 and haplotype 4, which are the risk for Japanese bipolar samples and NIMH trios, were significantly higher than that of haplotype 1 (Fig. 3A). Similar difference was observed during the stimulation by 300 nM thapsigargin, the ER Ca^{2+}-ATPase inhibitor. The response to ER stress did not differ between haplotypes. Co-transfection with spliced form XBP1-expressing vector enhanced the promotor activity. This enhancement was observed similarly in these four constructs (Fig. 3B).

Genotyping of the genomic DNA and mRNA in the brain samples

The result of genetic analysis in NIMH trios is compatible with a hypothesis that the haplotype 3 confers a risk only when it is paternally transmitted. A possible explanation for such a phenomenon, that is parent-of-origin effect, is genomic imprinting [2]. To test whether or not HSPA5 shows monoallelic expression in the brain, we genotyped the SNP7 at 3′-UTR in genomic DNA and cDNA obtained from postmortem brain tissues [prefrontal or frontal cortex of bipolar disorder ($n = 41$), schizophrenia ($n = 46$), depression ($n = 12$), and control ($n = 41$)]. The 81 samples having heterozygous genotype in genomic DNA also showed heterozygous genotype in cDNA, which did not suggest monoallelic expression.

Discussion

In this study, we found that there are four haplotypes in the promotor region of HSPA5. A promotor assay revealed that these polymorphisms affect the promotor activity. We also demonstrated that the haplotype of *HSPA5* (*GRP78/BiP*) gene was nominally associated with bipolar disorder in Japanese population. However, because (1) the global *p* value was not significant, (2) the association was not replicated in an independent sample set, and (3) no association was found in NIMH trio samples, this could be a type I error. Since the global *p* value was significant in the samples with family history of mood disorder, it would be interesting to test the association in an independent sample set of bipolar disorder with family history. Because parent-of-origin effect is suggested in bipolar disorder [2,32–34] although controversial [35–38], we hypothesized that parent-of-origin effect might confound the findings in NIMH trios. Thus, we performed the hypothesis-generating analysis, which showed significant over-transmission of haplotype 3 from paternal side. This finding made us to hypothesize that HSPA5 is subjected to genomic imprinting. However, monoallelic expression was not observed in the frontal cortex. This did not support our hypothesis. Because imprinting is dependent on the region of the brain [39,40], a possibility that HSPA5 is imprinted in the specific brain region cannot be totally excluded. However, before considering such possibility, this finding of nominal association in paternal transmission should be tested in an independent sample set.

The haplotype in the upstream region showing the nominal association in Japanese and NIMH altered the promotor activity. In this experimental condition, these haplotypes were associated with higher promotor activity. HSPA5 is known to have three ER stress response elements (ERSE, consensus sequence; CCAAT-N9-CCACG) (ERSE1–3) in the promotor region [41]. All the SNPs (SNP1–4) located not within but upstream of ERSE, and thus are unlikely to alter ER stress response. Indeed, there was no difference of response rate among haplotypes against ER stress induced by thapsigargin, while the risk haplotype showed higher activity both in the basal level and after induction of ER stress. In addition to ERSE, *HSPA5* expression is controlled by binding sites of many transcription factors such as ATF4 and AP-1 [42,43]. However, the three SNPs in the risk haplotype do not alter the binding sites of known transcription factors. Unknown transcription factor may determine the basal promotor

activity. Higher promotor activity of the risk is seemingly inconsistent with the reported evidence that valproate improves the ER stress response, and *HSPA5* response to ER stress was impaired in bipolar disorder [4]. This may be due to the cell-type difference or because we used artificial vectors having only promotor regions. It is also possible that higher basal promotor activity might paradoxically result in impaired ER stress response. Although precise mechanism is still unclear, altered regulation of *HSPA5* may contribute to the pathophysiology of bipolar disorder.

The role of ER stress response in the brain is little clarified. Recently, ER stress response was reported to be critical for trafficking of AMPA-type glutamate receptors [44,45]. GluR1 accumulated in the ER of mutant *Caenorhabditis elegans* lacking XBP1 or IRE1. On the other hand, the role of lithium and valproate in AMPA GluR1 receptor trafficking was reported [46]. Altered ER stress response system may contribute to the pathophysiology of bipolar disorder via altered trafficking of AMPA receptors.

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