Table 2. Case-Control Comparisons of SNPs in Extended Individuals

SNP No.	dbSNP	Subjects		Genotype Count (Frequency)			p^a	Allele Count (Frequency)		p^b	Odds Ratio (95% CI)
				GG	GA	AA		G	A		
SNP1	rs2433320	Patients	n = 278	178 (.64)	84 (.30)	16 (.06)	.004	440 (.79)	116 (.21)	.004	1.49 (1.13–1.96)
		Controls	n = 462	331 (.72)	123 (.27)	8 (.02)		785 (.85)	139 (.15)		
				TT	TC	CC		T	C		
SNP4	rs2433322	Patients	n = 278	178 (.64)	84 (.30)	16 (.06)	.004	440 (.79)	116 (.21)	.004	1.49 (1.13–1.96)
		Controls	n = 462	331 (.72)	123 (.27)	8 (.02)		785 (.85)	139 (.15)		
				CC	CT	TT		C	T		
SNP16	rs11732668	Patients	n = 272	81 (.30)	143 (.53)	48 (.18)	.02	305 (.56)	239 (.44)	.03	1.28 (1.03–1.59)
		Controls	n = 462	176 (.38)	221 (.48)	65 (.14)		573 (.62)	351 (.38)		
				AA	AG	GG		A	G		
SNP18	rs12641023	Patients	n = 272	49 (.18)	140 (.51)	83 (.31)	.06	238 (.44)	306 (.56)	.07	1.23 (0.99–1.52)
		Controls	n = 459	68 (.15)	220 (.48)	171 (.37)		356 (.39)	562 (.61)		

SNP, single nucleotide polymorphism; CI, confidence interval; Controls, control subjects.
[a]Armitage's trend test.
[b]Fisher's exact test.

manner. Such trafficking promotes synaptic accumulation of receptors (Schulz et al 2004). Many postsynaptic density proteins that contain PDZ domain(s) interact with glutamate receptors to control receptor dynamics and synaptic plasticity (Bach 2000; Fanning and Anderson 1999; Hayashi et al 2000; Jelen et al 2003; Remedios et al 2004). Taken together, these findings suggest that proteins with LIM domains and PDZ domains play important roles in synaptic development and plasticity.

In conclusion, our findings provide some evidence that the PDLIM5 gene might be involved in susceptibility to schizophrenia. Further studies of the involvement of the PDLIM5 gene in the pathophysiology of schizophrenia and confirmation of the present association in other populations are necessary.

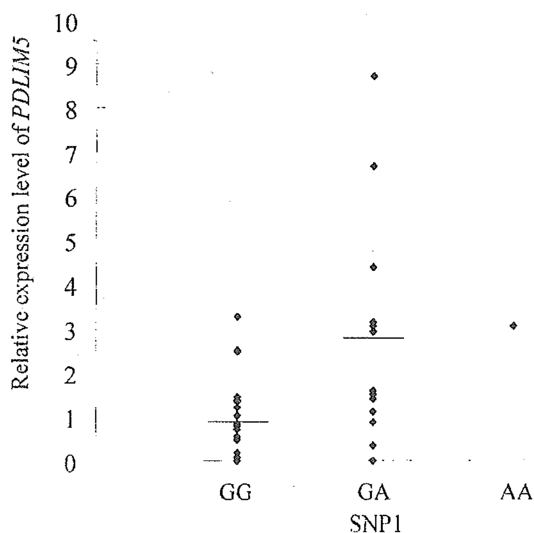

Figure 2. Expression of the PDZ and LIM domain 5 gene (PDLIM5) in postmortem brains from schizophrenia patients classified according to single nucleotide polymorphism (SNP)-1 (rs2433320) genotype. Expression of PDLIM5 was normalized to that of glyseraldehyde-3-phosphate dehydrogenase (GAPDH). The difference of expression between GG genotype and GA genotype is significant (two-tailed Student's *t* test, p = .007; Wilcoxon test, p = .008). GG: SNP 1-GG genotype (n = 20); GA: SNP 1 GA genotype (n = 13); AA: SNP 1 AA genotype (n = 1). The **horizontal line** indicates mean.

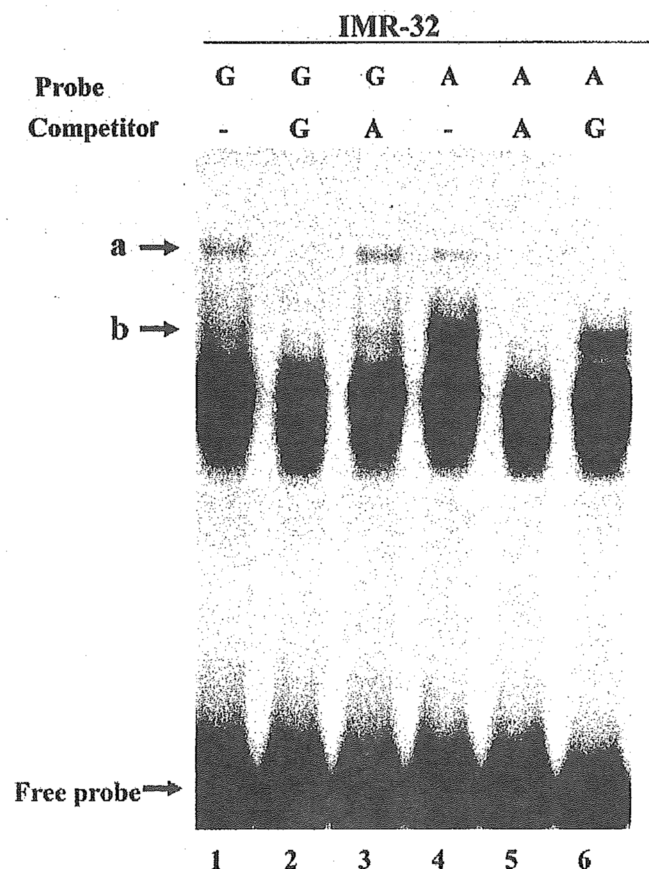

Figure 3. Electrophoretic mobility shift assay (EMSA) with the PDZ and LIM domain 5 gene (PDLIM5) single nucleotide polymorphism (SNP)-1 fragments. Nuclear proteins were extracted from IMR-32 cells. The PDLIM5 promoter fragments with either SNP1-G or SNP1-A were synthesized and used as DNA probes. Lane 1, SNP1-G without cold competitor; lane2, SNP-G with 100 fold excess of cold SNP1-G; lane 3, SNP-G with 100 fold excess of cold SNP1-A; lane4, SNP1-A without cold competitor; lane5, SNP-A with 100 fold excess of cold SNP1-A; lane 6, SNP-A with 100 fold excess of cold SNP1-G. **Arrows** indicate DNA-protein complex bands. Unbound probe is also indicated. The experiments were repeated three times with the same results.

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Association between chromogranin A gene polymorphism and schizophrenia in the Japanese population

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14 **Abstract**

15 It has been reported that expression of the chromogranin A (*CHGA*) gene is reduced in the prefrontal cortex and
16 cerebrospinal fluid of patients with schizophrenia. Single-marker and haplotype analyses of SNPs within the *CHGA* gene were
17 performed in 633 subjects with schizophrenia and 589 healthy controls. A significant association with schizophrenia was
18 observed to one SNP marker, rs9658635 ($p=0.0269$), and with a 2 marker haplotype ($p=0.0016$). Significant association of
19 rs9658635 was then replicated in a second independent cohort (377 schizophrenia and 338 control samples) ($p=0.007$). These
20 results suggest that the *CHGA* gene is associated with the risk of developing schizophrenia in the Japanese population.
21 © 2006 Published by Elsevier B.V.

22 *Keywords:* Chromogranin A; Schizophrenia; Gene; Haplotype; Association

23

1. Introduction

24

Numerous family and twin studies have suggested 25
that genetic factors contribute to the development of 26
schizophrenia. The gene encoding chromogranin A 27
(*CHGA*) is located on chromosome 14q32. Meta- 28
analysis of genome-wide studies and a genome-wide 29
study of the Japanese population (JSSLG, 2005) 30
showed evidence of linkage to 14q12-13, although no 31

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linkage to the *CHGA* region, 14q32, has been reported. However, a significant positive association between the AKT-1 gene, located in this region, and schizophrenia has been reported in the Japanese population (Ikeda et al., 2004).

Chromogranin A (CHGA), which is widely distributed in the central nervous system and is co-released with various neurotransmitters, is involved in the selective aggregation of regulated secretory proteins in the trans-Golgi network (Taupenot et al., 2003), the regulation of Ca^{2+} release from endoplasmic reticulum (Yoo, 2000), and in secretory granule biogenesis (Kim et al., 2001). Biochemical studies have demonstrated a reduction of CHGA immunoreactivity in the prefrontal cortex (Iwazaki et al., 2004) of schizophrenic patients. Miller et al. (1996) reported an increase in the peptide derived from CHGA but not other neurotransmitters, such as dopamine, norepinephrine, or serotonin, in the CSF of patients with drug-naïve first episodes of schizophrenia. Subsequently, Landen et al. (1999) reported a reduction in the levels of CHGA in the CSF of patients with chronic schizophrenia and suggested that an acute increase in the level of CHGA might reflect an active disease process.

These results suggest that altered function of CHGA might be related to the abnormality of synaptic connectivity, one of the major hypotheses explaining the pathophysiology of schizophrenia. Furthermore, significant associations have been reported between schizophrenia and the chromogranin B gene, which belongs to the same family as CHGA, in Japanese populations (Iijima et al., 2004).

In the present study, we performed linkage disequilibrium (LD) analysis of *CHGA*, and carried out case–control association studies of *CHGA* polymorphisms and schizophrenia using single-marker association analysis and haplotype analysis.

2. Methods

2.1. Subjects

This study was performed with approval from the ethics committee of Nagoya University. Written informed consent to participate was obtained from all subjects involved in this research. 641 unrelated schizophrenic patients (268 males and 373 females; mean age 51.7 ± 4.8 years) who met the criteria for schizophrenia of the DSM-III-R (Revised Third Version of the Diagnostic and Statistical Manual) and 582 healthy controls (290 males and 292 females; mean age 42.0 ± 4.8 years) were genotyped. A second independent set of samples, 377 unrelated patients with schizophrenia (193 males and 184 females; mean age 38.5 ± 5.5 years) and 338 healthy controls (183 males and 155 females; mean age 34.2 ± 3.7 years), was genotyped for confirmation of these positive results. None of the schizophrenic subjects in this study was included in the previous Japanese linkage study for schizophrenia (JSSLG, 2005). All subjects were of Japanese descent.

2.2. Genotyping

A total of six single nucleotide polymorphisms (SNPs; rs9658635, rs7159323, rs2295396, rs941584, rs729940, and rs875395) with minor allele frequencies 20% were selected, and the information on these SNPs was obtained from the dbSNP database (http://www.ncbi.nlm.nih.gov/SNP). Genotyping was carried out using polymerase chain reaction–restriction fragment length polymorphism assays or direct sequence assays for each SNP. Information about each primer and enzyme is available on request.

2.3. Sequencing analysis

Primer pairs were designed using information from the GenBank sequence (accession number: NT-026437). We used direct sequencing with a Big Dye Terminator Cycle Sequencing kit and ABI PRISM 3130 Genetic Analyzer (Applied Biosystems, Japan). Information on the sequencing of primer pairs is available on request.

2.4. Statistical analysis

Genotype deviation from the Hardy–Weinberg equilibrium was evaluated using the χ^2 test. Pairwise LD was evaluated among the possible combinations of each SNP. The LD block was defined as the region where the D' between each SNP was greater than 0.8, and was examined using the genotype data for 96 healthy controls with HAPLOVIEW version 3.0

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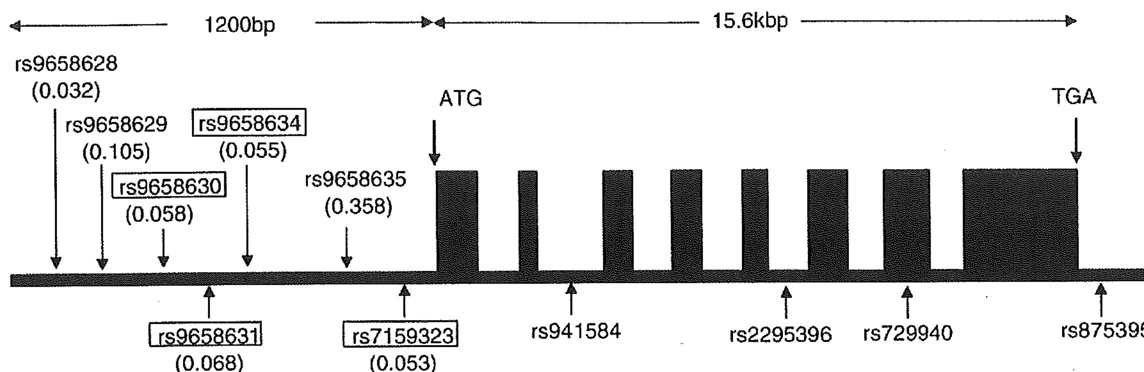

Fig. 1. Genomic structure of the human chromogranin (CHGA) and the approximate locations of the single nucleotide polymorphisms. The SNP IDs in the boxes refer to the SNPs studied by Wen et al. Numbers (in parenthesis) represent minor allele frequencies of 96 schizophrenic patients.

(http://www.broad.mit.edu/mpg/haploview/). These subjects were included in the association study. In each LD block, the haplotype frequency was estimated with the aid of the expectation–maximization algorithm, and the haplotype tag SNPs (htSNPs) were selected using the same program. Single-marker association analyses and haplotype analyses were performed using SPSS version 11.0J (Tokyo, Japan) and Cocaphase 2.403 (http://www.rfcgr.mrc.ac.uk/~fdudbrid/software/unphased/), respectively. The level of significance for all statistical tests was set at 0.05. For the correction of multiple comparisons, the permutation test (10,000 iterations) was performed.

3. Results

The position of SNPs and the structure of CHGA are shown in Fig. 1. It was found that CHGA was in a single block exhibiting strong LD (Fig. 2). The SNPs rs9658635 and rs729940 were selected as htSNPs. The genotype and allele frequencies of these two SNPs in schizophrenic

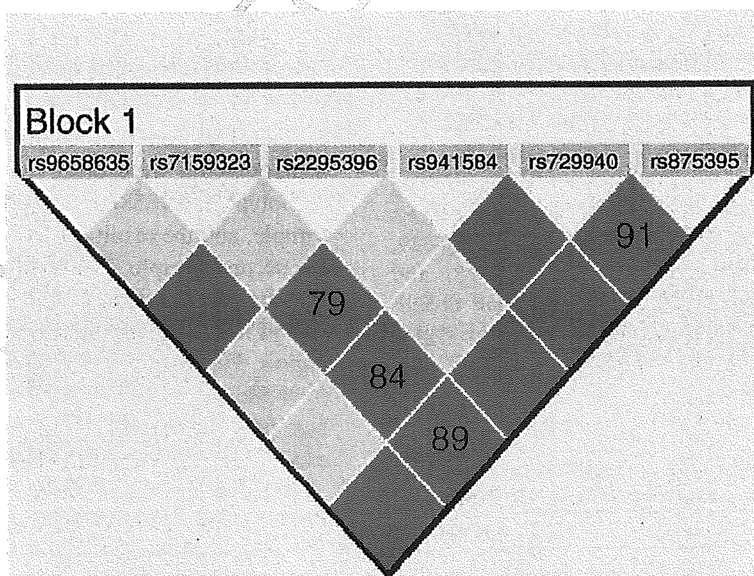

Fig. 2. Linkage disequilibrium between 6 SNPs of CHGA gene in 96 controls. Numbers in the box represent D' values after the decimal point. D' values of 1.0 are not shown.

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Table 1
Genotype and allele distributions of the *CHGA* SNPs in patients with schizophrenia and controls in the first sample set

SNP	Sample	Genotype[a]			Allele		*P* value	*P* value-corrected[b]
		WW	WM	MM	W	M		
rs9658635	Schizophrenia	284	266	72	834	410	0.010	0.0269
	Control	207	270	75	684	420		
rs729940	Schizophrenia	406	191	37	1003	265	0.066	
	Control	388	158	23	934	204		

[a] W, wild allele; M, minor allele.
[b] *P* value was corrected by 10,000 permutations test.

Table 2
Haplotype distributions of the *CHGA* in patients with schizophrenia and controls in the first sample set

Sample	Haplotype (rs9658635–rs729940)				*P* value[a]	*P* value-corrected[b]
	T–C	C–C	T–T	C–T		
Schizophrenia	0.4890	0.3010	0.1750	0.0350		
Control	0.4405	0.3771	0.1696	0.0128		
P value[c]	0.0636	0.0006	0.2364	0.0301	0.000612	0.0016

[a] *P* value was calculated by log-likelihood ratio test (global haplotype association).
[b] *P* value was corrected by 10,000 permutations test.
[c] *P* values were calculated by individual haplotype test.

patients and control subjects are summarized in Table 1. The observed genotype frequencies of all SNPs were within the normal distribution expected according to the Hardy–Weinberg equilibrium. Single-marker case–control association analyses revealed significant associations in allele frequencies for rs9658635 ($p=0.0269$). In addition, there was a significant difference in the distribution of haplotype frequencies between the schizophrenic and the control subjects ($p=0.0016$; Table 2). In the second sample set, a significant association with schizophrenia was replicated in allele frequency for rs9658635 ($p=0.007$) (Table 3).

In light of these results, mutations in the 5′ flanking regions of CHGA were searched for using 96 subjects with schizophrenia. Seven SNPs (rs9658628, rs9658629, rs9658630, rs9658631, rs9658634, rs9658635, and rs7159323) that had been already listed on the dbSNP database were detected. The positions of SNPs in this region are shown in Fig. 1. With the exception of rs9658629 and rs9658635, these SNPs were rarely present in the promoter region, the minor allele frequencies being lower than 10%.

Table 3
Genotype and allele distributions of the *CHGA* SNP in patients with schizophrenia and controls in the second sample set

SNP	Sample	*n*	Genotype			Allele		*P* value
			WW	WM	MM	W	M	
rs9658635	Schizophrenia	377	188	133	56	509	245	0.007
	Control	338	122	166	59	410	266	

4. Discussion

The results presented here suggest that *CHGA* is involved in the development of schizophrenia in the Japanese population. Significant associations with schizophrenia were observed for rs9658635 and the haplotype of rs9658635–rs729940 in *CHGA* in the first sample, and the result for rs9658635 was replicated in the second sample. The SNP rs9658635, for which a significant association with schizophrenia was observed in the present study, is located in the promoter region. However, the haplotype of four other SNPs (rs9658630, rs9658631, rs9658634, and rs7159323) has been reported to be associated with altered expression/function of the CHGA (Wen et al., 2004).

Because the two marker haplotypes are more significant than single markers alone, the combination of the two significant SNPs may produce functional changes. However, there is a possibility that there is a SNP in LD with rs729940 and rs9658635, and that this SNP may be more strongly associated with schizo-

177 phrenia. The effect of the SNP on the gene expression
178 and its role in increasing risk for schizophrenia remains
179 to be clarified.

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230

The Breakpoint Cluster Region Gene on Chromosome 22q11 Is Associated with Bipolar Disorder

Ryota Hashimoto, Takeya Okada, Tadafumi Kato, Asako Kosuga, Masahiko Tatsumi, Kunitoshi Kamijima, and Hiroshi Kunugi

Background: *Although the pathogenesis of bipolar disorder remains unclear, heritable factors have been shown to be involved. The breakpoint cluster region (BCR) gene is located on chromosome 22q11, one of the most significant susceptibility loci in bipolar disorder linkage studies. The BCR gene encodes a Rho GTPase activating protein, which is known to play important roles in neurite growth and axonal guidance.*

Methods: *We examined patients with bipolar disorder* ($n = 171$), *major depressive disorder* ($n = 329$) *and controls* ($n = 351$) *in Japanese ethnicity for genetic association using eleven single nucleotide polymorphisms (SNPs), including a missense one (A2387G; N796S), in the genomic region of BCR.*

Results: *Significant allelic associations with bipolar disorder were observed for three SNPs, and associations with bipolar II disorder were observed in ten SNPs including N796S SNP (bipolar disorder,* $p = .0054$*; bipolar II disorder* $p = .0014$*). There was a significant association with major depression in six SNPs. S796 allele carriers were in excess in bipolar II patients* ($p = .0046$, *odds ratio* $= 3.1$, *95% CI 1.53–8.76). Furthermore, we found a stronger evidence for association with bipolar II disorder in a multi-marker haplotype analysis* ($p = .0002$).

Conclusions: *Our results suggest that genetic variations in the BCR gene could confer susceptibility to bipolar disorder and major depressive disorder.*

Key Words: Breakpoint cluster region (BCR), bipolar disorder, major depression, 22q, association study, single nucleotide polymorphism (SNP)

Bipolar disorder is a major psychiatric disorder that is characterized by fluctuation between abnormal mood states of mania and depression. Since lithium has been one of the primary drugs used to treat bipolar disorder, molecular and cellular actions of this drug are believed to be clues of the pathophysiology of this disease, e.g. inhibition of glycogen synthase kinase-3, inositol monophosphatase, or N-methyl-D-aspartate (NMDA) receptor activity, activation of BDNF/Trk pathway, and enhancement of neurogenesis and neuronal progenitor proliferation (Chen et al 2000; Hallcher and Sherman 1980; Hashimoto et al 2002a, 2002b, 2003; Klein and Melton 1996). Recently, a common mechanism of action for three mood-stabilizing drugs, lithium, valproate and carbamazepine, has been identified (Williams et al 2002). These drugs inhibit the collapse of sensory neuron growth cones and increase growth cone area. Inositol reverses the effects of the drugs on growth cones, implicating inositol depletion in their action.

The breakpoint cluster region (BCR) gene is located on chromosome 22q, one of the most consistently replicated susceptibility loci in linkage studies of bipolar disorder (Detera-Wadleigh et al 1999; Edenberg et al 1997; Kelsoe et al 2001; Turecki et al 2001). A recent meta-analysis of eleven published genome scans for bipolar disorder revealed the strongest evidence for susceptibility loci on 22q and 13q (Badner and Gershon 2002). The BCR gene encodes a Rho GTPase-activating protein (GAP) highly expressed in hippocampal pyramidal cell layer and dentate gyrus (Fioretos et al 1995). The Rho family of GTP binding proteins acts as a key regulator for developing neuronal network, e.g. growth cone and neurite formation (Negishi and Katoh 2002). These proteins cycle between active GTP-bound and inactive GDP-bound forms. The activation of GTP-bound form is regulated by GAPs, which stimulate GTP hydrolysis, leading to inactivation (Etienne-Manneville and Hall 2002).

Therefore, genetic variability of the BCR gene is of considerable interest in the evaluation of risk of bipolar disorder. To our knowledge, however, there is no study examining the possible association between the BCR gene and bipolar disorder. The BCR gene (Online Mendelian Inheritance in Man [OMIM]:151410) consists of 23 exons and 22 introns, spanning 135 Kb. We searched for polymorphisms in the BCR gene in silico and selected eleven single nucleotide polymorphisms (SNPs), including a common single nucleotide substitution (A2387G; National Center for Biotechnology Information [NCBI] SNP ID: rs140504) in exon 10 giving rise to an amino acid change of asparagine to serine at codon 796 (N796S; NCBI Protein ID: NP_004318). In the present study, we performed an association study with SNPs in the region of the BCR gene in a Japanese population of bipolar and major depression cases and controls.

Methods and Materials

Subjects

Subjects were 171 patients with bipolar disorder (65 males and 106 females with mean age of 50.8 years [SD 14.9] and mean age of onset of 39.2 years [SD 15.2], 102 bipolar I [43 males and 59 females] and 69 bipolar II patients [22 males and 47 females]), 329 patients with major depressive disorder (116 males and 213 females with mean age of 54.3 years [SD 16.0] and mean age of onset of 46.7 years [SD 15.3]) and 351 healthy controls (170 males

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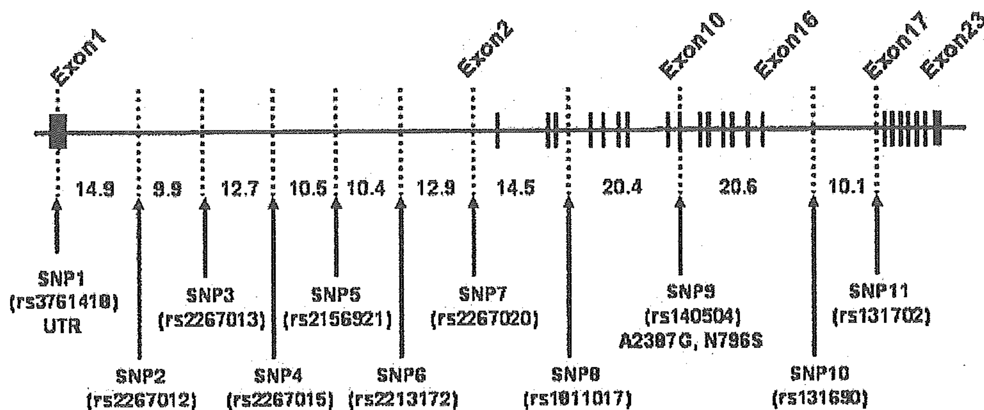

Figure 1. Genomic structure and location of single nucleotide polymorphisms (SNPs) for human Breakpoint Cluster Region (*BCR*) gene. Exons are denoted by bold vertical lines in black. The rs number of each SNP is the National Center for Biotechnology Information SNP cluster ID from the dbSNP database. The distances of the adjunct SNPs (Kb) are also shown.

and 181 females with mean age of 40.2 years [SD 12.0]). In addition, subjects who were examined in our previous study on the XBP1 gene (Kakiuchi et al 2003), were included in this study; 83 patients with bipolar disorder (27 males and 56 females with mean age of 48.1 years [SD 14.6] and mean age of onset of 35.9 years [SD 15.1], 57 bipolar I [23 males and 34 females] and 26 bipolar II patients [4 males and 22 females]) and 97 healthy controls (51 males and 46 females with mean age of 38.8 years [SD 13.4]). All the subjects were biologically unrelated Japanese. Consensus diagnosis was made for each patient by at least two trained psychiatrists according to the DSM-IV criteria, based on all available information, including clinical interview, medical records and other research assessments. Controls were healthy volunteers who had no current or past contact to psychiatric services. Subjects with significant medical problems, history of head trauma, neurosurgery and alcohol or substance abuse were excluded. After description of the study, written informed consent was obtained from every subject. The study protocol was approved by institutional ethical committees (Showa University School of Medicine, RIKEN Brain Science Institute and National Center of Neurology and Psychiatry, Tokyo, Japan).

SNP Genotyping

Venous blood was drawn from the subjects and genomic DNA was extracted from whole blood according to the standard procedures. Eleven SNPs (SNP1: rs3761418, SNP2: rs2267012, SNP3: rs2267013, SNP4: rs2267015, SNP5: rs2156921, SNP6: rs2213172, SNP7: rs2267020, SNP8: rs1811017, SNP9: rs140504, SNP10: rs131690, SNP11: rs131702; see figure 1) in the BCR gene were genotyped using the TaqMan 5'-exonuclease allelic discrimination assay, described previously (Hashimoto et al 2004; Hashimoto et al 2005). Briefly, primers and probes for detection of the SNPs are: SNP1: forward primer 5'-GGGAGTGAA-ACAAAATCTTTGATGGTT-3', reverse primer 5'-ATCAGACTC-CCTGCTCTTTGC-3', probe 1 5'-VIC-CTGTCTCAGATTTCCAG-MGB-3', and probe 2 5'-FAM-CTGTCTCAGATCTCCAG-MGB-3'; SNP2: forward primer 5'-GCATTTTGCAGAATGTCTTCTCTCA-3', reverse primer 5'-ACACTCAGCTAAGAGGGTTCCT-3', probe 1 5'-VIC-CCCTGTGAAGGAGTG-MGB-3', and probe 2 5'-FAM-CCTGTGGAGGAGTG-MGB-3'; SNP3: forward primer 5'-TCT-TTGTCAGCGCTGTGGTT-3', reverse primer 5'- CCCACAACAG-CAATAAACTAGCAAA-3', probe 1 5'-VIC- CAGTAAGTTCTTTC-CCTACCAAG-MGB-3', and probe 2 5'-FAM- TAAGTTCTTTC-CCCACCAAG-MGB-3'; SNP4: forward primer 5'-CCACCCT-AGGGCATTTTCCT-3', reverse primer 5'-CCAGCTTTCCACTGT-TATGAATACAATG-3', probe 1 5'-VIC-CCCCTTTTCTTTTATGG-TAG-MGB-3', and probe 2 5'-FAM-CCCCTTTTCTTTTTTGGTAG-MGB-3'; SNP5: forward primer 5'-GGAATAGCAGAGTAT-CTTTTCAACTAGGTT-3', reverse primer 5'-GGACTTCTGGC-CCCTTTCAG-3', probe 1 5'-VIC-CCCCTCAATTGCAC-MGB-3', and probe 2 5'-FAM-CCCCTCAGTTGCAC-MGB-3'; SNP6: forward primer 5'-CTAGCAGCTGTGCTCATGGA-3', reverse primer 5'-AGGCCCAGCTCCTATCCT-3', probe 1 5'-VIC-ATCTCACGTC-CTCCC-MGB-3', and probe 2 5'-FAM-AATCTCACCTCCTCCC-MGB-3'; SNP7: forward primer 5'-CTCGGTGTTGACTTGACCT-TACA-3', reverse primer 5'-GGTGGAGCACCTTTTATCTGAGT-3', probe 1 5'-VIC-CTTTCCGAGCCCATG-MGB-3', and probe 2 5'-TTTCCGCGCCCATG-MGB-3'; SNP8: forward primer 5'-GC-CACTTCCTGGAAAGAAAGGT-3', reverse primer 5'-TGAGGTCT-GGCTGGTGCTA-3', probe 1 5'-VIC-CTGCCAATAGCCC-MGB-3', and probe 2 5'-CTGCCAGTAGCCC-MGB-3'; SNP9: forward primer 5'-AGCTGGACGCTTTGAAGATCA-3', reverse primer 5'-TGGTGTGCACCTTCTCTCTCT-3', probe 1 5'-VIC-CCAGATCAA-GAATGACAT-MGB-3', and probe 2 5'-FAM-CCAGATCAAGAGT-GACAT-MGB-3'; SNP10: forward primer 5'-CCTGCCTGCCCAG-TCC-3', reverse primer 5'-CCCTGGGTTGCAAGGTCTT-3', probe 1 5'-VIC-CAGGCATATTCCTCA-MGB-3', and probe 2 5'-FAM-CAGGCATGTTCCTCA-MGB-3'; SNP11: forward primer 5'-CA-GACTGTGTTCGGGTGACA-3', reverse primer 5'-ACCCGGCAC-TATCCAGACA-3', probe 1 5'-VIC-CAGGAGCTTGTCCTTAA-MGB-3', and probe 2 5'-FAM-CAGGAGCTTGTCATTAA-MGB-3'. PCR cycling conditions were: at 95°C for 10 min, 45 cycles of 92°C for 15 sec and 60°C for 1 min.

Statistical Analysis

Statistical analysis of association studies was performed using SNPAlyse software (DYNACOM, Yokohama, Japan). The presence of Hardy-Weinberg equilibrium was examined using the χ^2 test for goodness of fit. Allele distributions between patients and controls were analyzed by the χ^2 test for independence. The measures of linkage disequilibrium (LD), denoted as D' and r^2, were calculated from the haplotype frequency using Expectation-Maximization algorithm. Case-control haplotype analysis was performed by the permutation method to obtain empirical significance (Good 2000). The global p-values represent the overall significance using the χ^2 test when the observed versus expected frequencies of all the haplotypes are considered together. The individual haplotypes were tested for association by

grouping all others together and applying the χ^2 test with 1 *df.* Calculations of *p*-values were based on 10,000 replications. All p-values reported are two tailed. Statistical significance was defined at $p < .05$.

The population homogeneity was assessed using STRUCTURE software (http://pritch.bsd.uchicago.edu/software.html) (Pritchard et al 2000) with eight SNPs, as described previously (Yamada et al 2004). In the application of the Markov chain Monte Carlo method, 1,000,000 replications were used for the burn-in period of the chain and for parameter estimation. Analysis was run at K = 1, 2, 3, 4, and 5. From these results, best estimate of K was found by calculating posterior probabilities, Pr (K = 1, 2, 3, 4, or 5).

Results

The genotype distributions of all the eleven SNPs were in Hardy-Weinberg equilibrium for both the controls and patients with bipolar disorder and major depressive disorder (data not shown). Allele frequencies of the eleven SNPs among the patients and controls are shown in Table 1. The minor alleles of SNP9, SNP10 and SNP11 were in excess in our total bipolar patients when compared to controls (SNP9: $\chi^2 = 7.73$, $df = 1$, $p = .0054$, odds ratio = 1.45 95% CI 1.11–1.84; SNP10: $\chi^2 = 7.48$, $df = 1$, $p = .0063$, odds ratio = 1.50 95% CI 1.14–2.03; SNP11: $\chi^2 = 9.05$, $df = 1$, $p = .0026$, odds ratio = 1.49 95% CI 1.15–1.93), while significant association of the other eight SNPs was not observed with the overall bipolar patients (Table 1). When we examined bipolar I and II separately, there were significant differences in the allele frequency for ten SNPs between patients with bipolar II disorder and controls, while there was a significant difference for one SNP between those with bipolar I disorder and controls (Table 1). Then, we examined a possible association between major depression and the BCR gene. A significant difference in the allele frequency was found for six SNPs between patients with major depressive disorder and controls, and for seven SNPs between total patients with mood disorders and controls (Table 1).

We then focused on the association between the SNPs in the BCR gene and bipolar II disorder. The frequencies of minor allele carriers of the eleven SNPs were compared with major allele homozygotes, as we assumed that minor alleles might have a dominant effect for developing the disease. Nine out of eleven SNPs were significantly associated with bipolar II disorder (Table 2). The smallest *p*-value was obtained in SNP9, N796S missense polymorphism. The S796 allele was significantly more frequent in the bipolar II patients when compared to the controls ($\chi^2 = 8.58$, $df = 1$, $p = .0046$, odds ratio = 3.1, 95% CI 1.53–8.76).

To further analyze the haplotype structure in Japanese population, we computed D' and r^2 values for all combinations of the eleven SNPs spanning the BCR locus at an average density of 12.3kb (Table 3). Forty-nine of 55 of possible 2-marker haplotype analysis for all combinations of the eleven SNPs yielded globally significant evidence for association ($p < .05$). That high population of the associated haplotypes is not surprising given the nonindependence of the markers, suggesting that Bonferroni correction might not be appropriate. Adjacent combinations of up to ten markers were examined for association with bipolar II disorder. Six of the nine possible 3-marker haplotype revealed significant evidence for association, as did six of eight of the 4-marker haplotypes and four of seven of 5-marker haplotypes. In total, more than 70% of all possible haplotypes showed the results that gave global significance at $p < .05$. Notably, all the possible combinations of haplotypes including SNP9 (N796S) were associated with bipolar disorder.

Table 1. Allele Distributions for 11 SNPs in the BCR Gene Among Patients With Bipolar Disorder and With Major Depression and Controls

SNP-ID	SNP	Controls	BP			BPI			BPII			MDD			Total cases		
		n = 351	*n* = 171	*p* Value	OR	*n* = 102	*p* Value	OR	*n* = 69	*p* Value	OR	*n* = 329	*p* Value	OR	*n* = 500	*p* Value	OR
SNP1	A/G	.349	.395	ns	—	.343	ns	—	.471	.0066	1.66	.415	.012	1.32	.408	.014	1.29
SNP2	A/G	.405	.456	ns	—	.402	ns	—	.536	.0042	1.70	.474	.0097	1.33	.468	.0095	1.29
SNP3	A/G	.261	.295	ns	—	.230	ns	—	.391	.0018	1.82	.310	.044	1.27	.305	.047	1.24
SNP4	T/A	.289	.319	ns	—	.245	ns	—	.428	.0013	1.84	.347	.023	1.30	.337	.037	1.25
SNP5	A/G	.292	.319	ns	—	.245	ns	—	.428	.0017	1.81	.347	.031	1.29	.337	.050	1.23
SNP6	G/C	.298	.330	ns	—	.255	ns	—	.442	.0009	1.87	.353	.031	1.28	.345	.040	1.24
SNP7	G/T	.423	.477	ns	—	.436	ns	—	.536	.0144	1.58	.438	ns	—	.451	ns	—
SNP8	A/G	.051	.061	ns	—	.064	ns	—	.058	ns	—	.061	ns	—	.061	ns	—
SNP9	A/G	.481	.573	.0054	1.45	.534	ns	—	.630	.0014	1.84	.523	ns	—	.540	.018	1.26
SNP10	A/G	.218	.295	.0062	1.50	.270	ns	—	.333	.0036	1.79	.236	ns	—	.256	ns	—
SNP11	T/G	.393	.491	.0026	1.49	.480	.026	1.43	.507	.013	1.59	.386	ns	—	.422	ns	—

Minor allele frequencies in controls are shown. OR, odds ratio; BP, bipolar disorder; MDD, Major depressive disorder; SNP, single nucleotide polymorphism; BCR, breakpoint cluster region.

Table 2. Genotype Distributions for the SNPs in the BCR Gene Among the Patients With Bipolar II and Controls

	Controls			BPII			2/2 and 1/2 vs. 1/1	
SNP-ID	1/1	1/2	2/2	1/1	1/2	2/2	*p* Value	OR (95% CI)
SNP1	151 (43.0%)	155 (44.2%)	45 (12.8%)	19 (27.5%)	35 (50.7%)	15 (21.7%)	.017	1.99 (1.16–3.84)
SNP2	123 (35.0%)	172 (49.0%)	56 (16.0%)	15 (21.7%)	34 (49.3%)	20 (29.0%)	.031	1.94 (1.10–3.78)
SNP3	190 (54.1%)	139 (39.6%)	22 (6.3%)	26 (37.7%)	32 (46.4%)	11 (15.9%)	.012	1.95 (1.19–3.49)
SNP4	171 (48.7%)	157 (44.7%)	23 (6.6%)	22 (31.9%)	35 (50.7%)	12 (17.4%)	.010	2.03 (1.19–3.61)
SNP5	170 (48.4%)	157 (44.8%)	24 (6.8%)	22 (31.9%)	35 (50.7%)	12 (17.4%)	.012	2.01 (1.13–3.65)
SNP6	165 (47.0%)	163 (46.4%)	23 (6.6%)	21 (30.4%)	35 (50.7%)	13 (18.8%)	.011	2.03 (1.19–3.61)
SNP7	115 (32.8%)	175 (49.9%)	61 (17.4%)	15 (21.7%)	34 (49.3%)	20 (29.0%)	.070	1.75 (1.01–3.62)
SNP8	315 (89.7%)	36 (10.3%)	0 (0%)	61 (88.4%)	8 (11.6%)	0 (0%)	ns	—
SNP9	91 (25.9%)	182 (51.9%)	78 (22.2%)	7 (10.1%)	37 (53.6%)	25 (36.2%)	.0046	3.10 (1.53–8.76)
SNP10	215 (61.3%)	119 (33.9%)	17 (4.8%)	32 (46.4%)	28 (40.6%)	9 (13.0%)	.022	1.83 (1.08–3.19)
SNP11	128 (36.5%)	170 (48.4%)	53 (15.1%)	15 (21.7%)	38 (55.1%)	16 (23.2%)	.018	2.07 (1.20–4.02)

Allele 1 represents a major allele in each SNP. SNP, single nucleotide polymorphism; BCR, breakpoint cluster region; BP, bipolar disorder; OR, odds ratio.

Adjacent marker combinations yielding global evidence for association at $p < .005$ are presented in figure 2. The haplotypes that yielded global evidence for significant association at this level included the SNP9 and SNP10. The haplotypes that yielded the strongest global evidence for significant association consisted of markers SNP8-SNP9-SNP10-SNP11 (global permutation p value = .00041, 100,000 simulations). Given this result, we tested the contribution of individual haplotypes to the global result. The lowest p-value was obtained for the difference in the frequency of 1-2-2-2 haplotype (SNP8-SNP9-SNP10-SNP11), which was enriched in patients with bipolar II disorder compared with controls (estimated frequencies: patients 30.2% vs controls 16.7%, permutation p value = .0002). Another individual haplotype that yielded the evidence for significant association was 1-1-1-1 haplotype, which occurs at a frequency of 33.5% in the patients and 45.7% in the controls (permutation p value = .0066).

Discussion

We found a significant association between genetic variations of the BCR gene and bipolar disorder and major depressive disorder in a Japanese population. Our data suggest that the BCR gene is associated with bipolar II rather than bipolar I disorder. The weaker association with major depression compared to bipolar II disorder might be due to some patients with major depression who could develop bipolar II disorder in the future and/or the susceptibility both for bipolar II and major depression. The diagnostic category of bipolar II disorder is defined as less severe manic symptoms compared with bipolar I disorder. Bipolar II patients tend to have more previous episodes, including both depressive and hypo-manic, but less hospitalization and psychotic symptoms (Vieta et al 1997). Some studies showed that bipolar disorder is likely to be a quantitative trait (bipolar I – bipolar II – major depression), while other studies argued that bipolar II may be genetically distinct to bipolar I disorder (Kelsoe 2003). Bipolar I and bipolar II were of approximately similar prevalence in the first degree relatives of bipolar I probands (8.5 vs. 6.1%, respectively), while bipolar II was significantly more prevalent among the first degree relatives of bipolar II probands (3% vs. 30%, respectively) (Coryell et al 1984). These data suggest that some genes confer susceptibility to both bipolar I and bipolar II, while a separate and more common set of genes predisposes preferentially or exclusively to bipolar II disorder. Kelsoe proposed a model of bipolar genetics that some of the genes involved are specific for each of the phenotypes considered bipolar I, bipolar II and major depression and others are less specific and may predispose individuals to either bipolar disorders or major depression (Kelsoe 2003).

The XBP1 gene, which has been reported to be associated with bipolar disorder, is also located on the chromosome 22q (Kakiuchi et al 2003). However, the physical distance between the XBP1 and BCR gene is approximately 5.5 Mb, and the D' value between the -116C/G polymorphism of the XBP1 gene and

Table 3. Marker-to-Marker LD for All the Combinations of the 11 SNPs in the BCR Gene

—	SNP1	SNP2	SNP3	SNP4	SNP5	SNP6	SNP7	SNP8	SNP9	SNP10	SNP11
SNP1	—	.766^a	.610^a	.483^a	.493^a	.480^a	.311^a	.000	.240^b	.002^b	.067^a
SNP2	.985^a	—	.475^a	.576^a	.585^a	.557^a	.360^a	.001	.279^a	.009^b	.081^a
SNP3	.963^a	.957^a	—	.785^a	.801^a	.765^a	.148^a	.001	.114^b	.026^a	.015^a
SNP4	.797^a	.981^a	.952^a	—	.986^a	.946^a	.192^a	.001^b	.154^b	.041^a	.018^b
SNP5	.800^a	.981^a	.968^a	1.000^a	—	.959^b	.191^a	.001^b	.152^b	.039^a	.018^a
SNP6	.779^a	.945^a	.959^a	.993^a	.993^b	—	.193^a	.001^a	.153^b	.040^a	.016^b
SNP7	.653^a	.624^a	.554^a	.588^a	.582^a	.578^a	—	.040^a	.693^a	.113^a	.360
SNP8	.095	.137	.089	.086^b	.078^b	.106^a	1.000^a	—	.058^a	.000	.006
SNP9	.644^b	.617^a	.549^b	.593^b	.585^b	.579^b	.937^a	1.000^a	—	.118^a	.344^b
SNP10	.064^b	.147^b	.183^a	.244^a	.241^a	.247^a	.546^a	.105	.627^a	—	.430^a
SNP11	.285^a	.291^a	.165^a	.169^b	.170^a	.154^b	.638	.424	.702^b	1.000^a	—

For each pair of markers, the standardized D' is shown below the diagonal, and r^2 above the diagonal.
LD, linkage disequilibrium; SNP, single nucleotide polymorphism; BCR, breakpoint cluster region.
$^a p < .05$.
$^b p < .01$.

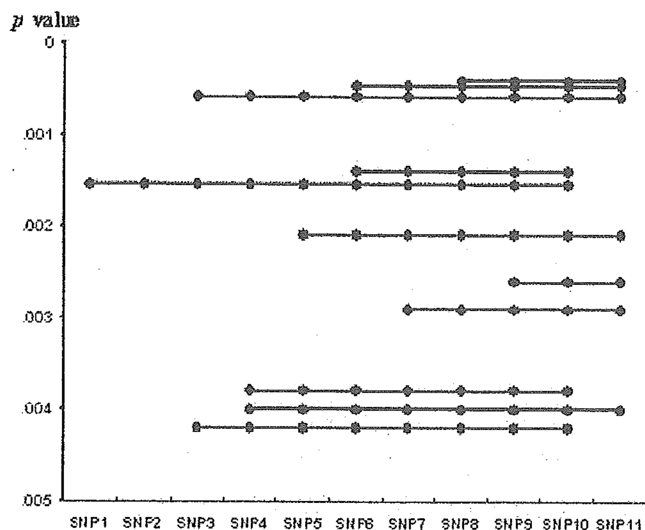

Figure 2. Plot of haplotypes showing global association to bipolar II disorder ($p < .005$). The x-axis scale is nonlinear in order to allow easy visualization of the different haplotypes. SNP, single nucleotide polymorphism.

any SNPs in the BCR gene is less than .3, and r^2 values are less than .004 both in patients and controls groups, suggesting that genetic association between bipolar disorder and both genes might be independent. In addition, we did not observe the evidence for the association between the -116C/G SNP and bipolar disorder in the population of the present study, although approximately half of the subjects in this study were overlapped with the Kakiuchi's study (G allele frequencies, Kakiuchi's, case: .71, control: .64; present study, case: .68, control: .70) (Kakiuchi et al 2003).

Our data suggest the existence in our sample of a risk haplotype and a protective haplotype consisting of SNP8-SNP9-SNP10-SNP11. We intended to determine the haplotype block structure of the region (Gabriel et al 2002), examining eleven SNPs at an average density of 12.3kb across the BCR gene. Unfortunately, we were not able to define the obvious haplotype blocks in a Japanese sample, because of the complex results of marker to marker LD. It is of interest how genetic variation might affect BCR function/expression. Although we have no evidence whether any of the SNPs in our haplotypes are functional, SNP9 associated with bipolar disorder might be functional as this SNP gives rise to an amino acid substitution of N796S in the functional domain, pleckstrin homology (PH) domain, of the BCR protein. This SNP might account for the susceptibility for bipolar disorder, as the individuals carrying the S796 allele were most significantly in excess in bipolar II patients in our study ($p = .0046$, odds ratio $= 3.1$). Alternatively, an unknown functional polymorphism, which is in LD with the SNPs and/or haplotypes, may be responsible for biologic susceptibility for bipolar II. Further work, e.g. dense mapping in the BCR gene, and functional analysis of N796S missense polymorphism, will be required to resolve this issue.

The function of the normal BCR gene product remains unclear, although the BCR-ABL fusion protein, which causes certain human leukemias, has been extensively studied (Pane et al 2002). The BCR gene encodes a 1271 amino acid protein containing several functional domains: a serine/threonine protein kinase domain, a Dbl homology domain, a PH domain and a Rho GTPase-activating protein domain. This protein acts as a serine/threonine kinase, a GTPase-activating protein for p21rac, and a Rho GTPase guanine nucleotide exchange factor (Diekmann et al 1991; Korus et al 2002; Maru and Witte 1991). The PH domain is a 100–120 amino acid protein module best known for its ability to bind phosphatidylinositol (Lemmon et al 2002). PH domain-containing proteins specifically recognize 3-phosphorylated phosphatidylinositol, allowing them to drive membrane recruitment in response to phosphatidylinositol 3-kinase activation. A dysfunction in the phosphatidylinositol signal transduction pathway appears to be implicated in the pathophysiology of bipolar disorder, e.g. increased intracellular calcium responsiveness and protein kinase C activity in platelets and transformed lymphoblasts, and decreased inositol levels in frontal cortex of the postmortem brain (Shimon et al 1997; Soares and Mallinger 1997). The N796S missense polymorphism in the PH domain of BCR could affect its binding activity to phosphatidylinositol, and then alter the phosphatidylinositol signal transduction pathway. Both asparagine and serine residue are neutral amino acids (polar amino acids), while only serine residue could be phosphorylated. Additionally, the sequence around N796 is evolutionarily conserved across several species, including fruit fly, African clawed frog, mouse, rat, and human. As S796 is unique for humans, this polymorphism might be associated with the higher brain function in humans.

As a common action of mood stabilizers is to inhibit the collapse of neuronal growth cone via depletion of inositol (Williams et al 2002), neuronal growth cone formation is likely to be involved in the pathogenesis of bipolar disorder. BCR is a RhoGAP protein, which inactivates the Rho GTPase. Rho GTPase proteins activate their effecters, which control cytoskeletal organization (Kaibuchi et al 1999), leading to the motile behavior of the neurite and growth cone (Huber et al 2003). As Rho associated kinase, one of the Rho effectors, regulates the dynamic reorganization of cytoskeletal proteins, such as actin, neurofilament and glial fibrillary acidic protein (Amano et al 1997; Hashimoto et al 1998; Kosako et al 1997), it is worth investigating the possible effect of BCR, including N796S amino acid change, on the growth cone formation and Rho associated kinase.

We have firstly demonstrated the possible association between the BCR gene and bipolar disorder in a Japanese population. The limitation of this work is that there were differences with regard to mean age and gender distribution between patients and controls. Thus, we assessed the population homogeneity and did not detect any evidence for such stratification in our samples with a Pr (K = 1) > .99. However, a false-positive association due to the small sample size of the bipolar II disorder patients could not be excluded in our study. Further investigations are warranted to confirm our findings in other samples.

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ORIGINAL ARTICLE

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The Gem interacting protein (GMIP) gene is associated with major depressive disorder

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Abstract Major depressive disorder (MDD) is a mood disorder with a significant heritable component. Structural neuronal impairment has been considered to be implicated in MDD, as it leads to brain morphological alterations such as hippocampal atrophy. The Gem interacting protein, GMIP, is a novel Rho GTPase-activating protein known to play important roles in neurite growth and axonal guidance. We examined the GMIP gene for possible association in a Japanese sample of 164 patients with MDD and 164 controls matched for sex. We found a significant association with MDD for one single nucleotide polymorphism (SNP) (−525G/A) located on the 5′-upstream region of the GMIP gene (p=0.039, odds ratio 1.66, 95% CI 1.05–2.69) and stronger evidence for association in a multimarker haplotype analysis (p=0.004). We then performed a promoter-luciferase reporter assay; the promoter activity for −525A allele, which was in excess in the MDD patients, was significantly decreased compared with the −525G allele in transient transfection experiments using three types of cell lines. Our results suggest that genetic variations in the GMIP gene can confer susceptibility to MDD, and the associated promoter SNP might play a role in the transcriptional regulation of the GMIP gene. Further study needs to be undertaken to validate the association between the GMIP gene and MDD.

Keywords Gem interacting protein (GMIP) · Major depressive disorder · Rho GTPase-activating protein · Single nucleotide polymorphisms (SNPs) · Luciferase reporter assay

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Introduction

Major depressive disorder (MDD) is a mood disorder that strikes a large proportion of the population. It is a complex disorder with unknown etiology, likely the result of the interplay between vulnerability genes and environmental stressors [4]. MDD has traditionally been considered to have a neurochemical basis, but recent studies have associated this complex disorder with volume reductions in brain structures and in the numbers and/or sizes of glia and neurons in discrete brain areas [12]. Although the precise cellular mechanisms underlying these morphometric changes are unknown, the increasing data indicate that MDD are associated with impairments of structural plasticity.

The Rho family of GTP-binding proteins act as a key regulator for developing neuronal structure, e.g., neurite and growth cone formation [14]. These proteins cycle between active GTP-bound and inactive GDP-bound forms. The activation of the GTP-bound form is regulated by GTPase-activating proteins, which stimulate GTP hydrolysis, leading to inactivation [6]. The Gem interacting protein gene, GMIP, was identified as a novel GTPase-activating protein, which binds to Gem, a member of the Ras superfamily of GTPases [1]. The human GMIP gene is localized to chromosome 19p11–12 and consists of 21 exons and 20 introns, spanning 14.13 kb. The GMIP mRNA of 3,840 bp, which is expressed ubiquitously, gives rise to a protein of 970 amino acids [1]. GMIP interacts with Gem through its N-terminal half and has a

Rho GTPase-activating protein domain in its C-terminal half. GMIP is able to inhibit RhoA function, leading to the actin cytoskeletal reorganization in vivo. Therefore, genetic variability of the GMIP gene is of considerable interest in the evaluation of the risk of MDD. To our knowledge, however, there is no study examining the possible association between the GMIP gene and MDD. In the present study, we performed an association study with single nucleotide polymorphisms (SNPs) in the region of the GMIP gene in a Japanese population of MDD cases and control subjects and analyzed the function of disease-associated SNP on transcriptional regulation.

Materials and methods

Subjects

Subjects were 164 patients [59 males, mean age of 49.5 years (SD 12.7)] with MDD and 164 healthy controls matched for sex [59 males, 47.4 years (SD 9.5)]. The mean ages in males are 47.2 years (SD 11.4), from 24 to 76 years old in MDD patients, and 45.1 years (SD 6.2), from 38 to 62 years old in controls; the mean ages in females are 50.8 years (SD 13.2), from 17 to 82 years old in MDD patients, and 48.6 years (SD 10.7), from 31 to 76 years old in controls. All the subjects were biologically unrelated Japanese and recruited from the same geographical area (the Western part of Tokyo Metropolitan). Consensus diagnosis was made for each patient by at least two psychiatrists according to the *Diagnostic and Statistical Manual of Mental Disorders, 4th edition* (DSM-IV) criteria on the basis of unstructured interviews and information from medical records. Among the 164 patients, 96 (59%) individuals had recurrent depressive episodes, and the remainder had a single episode. Eighty (49%) subjects had a history of admission to a psychiatric hospital, and 46 (28%) had a history of attempted suicide. The mean age of onset was 42.2 years (SD 12.7). The controls were healthy volunteers recruited from hospital staffs. They were interviewed, and those individuals who had a current or past history of psychiatric treatment were not included in the study. Subjects with significant medical problems, history of head trauma, neurosurgery, and alcohol or substance abuse were excluded. After a description of the study, a written informed consent was obtained from every subject. The study protocol was approved by institutional ethical committees.

SNP genotyping

Venous blood was drawn from the subjects, and genomic DNA was extracted from whole blood according to the standard procedures. We searched and selected validated SNPs in allele frequency in the genomic region of the GMIP gene-distributed 3- to 6-kb interval in silico. Four SNPs (SNP1, rs3794996; SNP2, rs2043293; SNP3, rs2304129; and SNP4, rs880090) in the GMIP gene and two SNPs outside of the genomic region of GIMP (SNP5, rs2288865 and SNP6, rs1054685) (Fig. 1) were genotyped using the TaqMan 5′-exonuclease allelic discrimination assay previously described [9, 15]. Briefly, primers and probes for the detection of the SNPs are: SNP1: forward primer 5′-TCCCTGGTGTGCTGTAATTGG-3′, reverse primer 5′-CCCTTCCGTGACCCTCAAAG-3′, probe 1 5′-VIC-CCTTGAACCTGATCCAG-MGB-3′, and probe 2 5′-FAM-CTTGAGCCTGATCCAG-MGB-3′; SNP2: forward primer 5′- GGGAGCTGTGAGGCTGTAG-3′, reverse primer 5′-TGCTATGGATGTCTTCCCTAAGC-3′, probe 1 5′-VIC-CCAGACCAGCCCAGTG-MGB-3′, and probe 2 5′-FAM-CCAGACCAGACCAGTG-MGB-3′; SNP3: forward primer 5′-GCGGGACGGAGTGTGA-3′, reverse primer 5′-TCAAGGGTCAGCAAAGGTCATTT-3′, probe 1 5′-VIC-TCATTAACCCAGGCCACA-MGB-3′, and probe 2 5′-CATTAACCGAGGCCACA-MGB-3′; SNP4: forward primer 5′-ACCACCCTGGCACCTTAAATAAG-3′, reverse primer 5′-TGGTGGGAGGTAGGGATATAT GG-3′, probe 1 5′-VIC-TCCGTGTTCACAATC-MGB-3′, and probe 2 5′-FAM-TCCGTCTTCACAATC-MGB-3′; SNP5: forward primer 5′-CCTCGCATGTGCCCACTA-3′, reverse primer 5′-GCATTTCCTGAGCTCTGACCAT-3′, probe 1 5′-VIC-ATGGAAGGCCCTGTTC-MGB-3′, and probe 2 5′-FAM-AATGGAAGGTCCTGTTC-MGB-3′; and SNP6: forward primer 5′-AGGTTTGTGGCTCCTTG CA-3′, reverse primer 5′-CCCCGTGGACTGCTTCAA-3′, probe 1 5′-VIC-CTGTCCCCGATAGAA-MGB-3′, and probe 2 5′-FAM-CTGTCCCCAATAGAA-MGB-3. PCR cycling conditions were at 95°C for 10 min, 45 cycles of 92°C for 15 s, and 60°C for 1 min.

Polymorphism screening in the 5′-upstream region of the GMIP gene

To detect sequence variations in the 5′-upstream region of the human GMIP gene, we initially sequenced DNA samples from 24 control subjects that have a 95% power

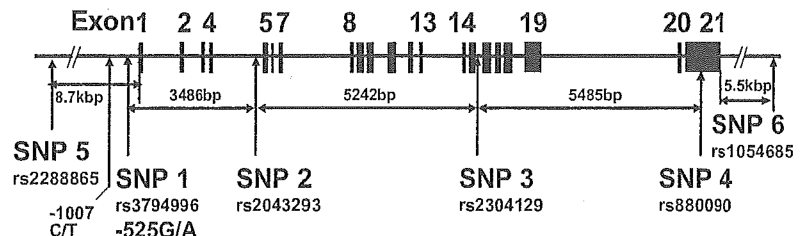

Fig. 1 Genomic structure and location of SNPs for human GMIP gene. Exons are denoted by *bold vertical lines in black*. The rs number of each SNP is the NCBI SNP cluster ID from the dbSNP database. The distances of the adjunct SNPs are also shown

to detect polymorphisms with a frequency of more than 5% [11]. Using three sets of GMIP-specific primers (forward primer 1 5′-CATCACAAGGTCAGGAGATCGA-3′, reverse primer 1 5′-CAGTGGAATTTGGGGCTGGAA-3′, forward primer 2 5′-GGAAACCTGGCTTGGCTCTTA-3′, reverse primer 2 5′-GGCCTGATATTCTGTCCGATTC-3′, forward primer 3 5′-CAAACCTCCACTCCCTACCT-3′, and reverse primer 3 5′-GTCCTTCCCATTCAGGAACT-3′), designed on the basis of the published GMIP mRNA sequences (accession number AF132541), we amplified, by PCR overlapping, fragments of the putative promoter region and exon 1 (from position −1,120 to +160 relative to the transcription start site). PCR, using primer 1 or 2, was performed under the following conditions: at 95°C for 10 min, 35 cycles of 95°C for 45 s, 62°C for 30 s and 72°C for 1 min, and 72°C for 7 min. PCR, using primer 3, was performed under the following conditions: at 95°C for 10 min, 45 cycles of 95°C for 45 s, 58°C for 30 s and 72°C for 1 min, and 72°C for 7 min. Individual PCR products were fully sequenced by a CEQ8000 autosequencer (Beckman Coulter, Fullerton, CA, USA) and compared with the original deposited sequence to identify variants.

Genotyping of the −1,007C/T SNP was performed using a PCR-based restriction-fragment-length polymorphism assay. Five hundred twenty seven base pair fragments were amplified using primers 5′-CATCACAAGGTCAGGAGATCGA-3′ and 5′-CAGTGGAATTTGGGGCTGGAA-3′, and then the product was digested by *Taq*I. The amplified T allele variant was cleaved twice, whereas the C allele was cleaved once, which was visualized by 2% agarose gel electrophoresis and ethidium bromide staining.

Luciferase reporter assay

Two allelic forms of the SNP1 (−525G/A) were cloned into pGL3-Basic vector (Promega, Madison, WI, USA). The fragments containing the −525G/A SNP were amplified by PCR from genomic DNA using the primers 5′-AAAGGGCTGCTCCCTGGTGTG-3′ (5′ end at position −564, transcriptional start site as +1) and 5′-ATATCTGGGCCCGGGGATCG-3′ (5′ end at position +81). These primers were designed to incorporate *Nhe*I (forward) and *Hin*dIII (reverse) restriction sites, and the PCR product was inserted into the polylinker site upstream of the luciferase coding region in the pGL3-Basic vector. The inserted sequence was confirmed with the autosequencer CEQ8000 in both directions using primers supplied by the manufacturer (Promega; RV primer 3 and GL primer 2).

Human neuroblastoma IMR-32 cells and HeLa cells were cultured in Minimum Essential Medium (Sigma, St. Louis, MO, USA) containing 10% fetal bovine serum (Invitogen, Carlsbad, CA, USA) and penicillin/streptomycin (Sigma). Human glioma Hs683 cells were cultured in Dulbecco's Modified Eagle Medium (Sigma) containing 10% fetal bovine serum and penicillin/streptomycin. Cells in 24-well plates were cotransfected in triplicate with 800 ng of pGL3-Basic firefly luciferase reporter vectors that included two allelic forms of the −525G/A SNP and 25 ng of Renilla luciferase expression vector (phRL-TK vector; Promega) as an internal control by using Lipofectamine 2000 Regent (Invitogen). As a negative control, an empty pGL3-Basic vector was transfected simultaneously in all the experiments. Cells were washed with phosphate-buffered saline and then harvested with luciferase lysis buffer (Promega) 24 h after transfection. A luciferase reporter assay was performed using a Dual-Luciferase Reporter Assay System (Promega) and a Lumat LB 9507 luminometer (Berthold, Bad Wildbad, Germany). Firefly and renilla luciferase activities were quantified sequentially as relative light units (RLU) by the addition of their respective substrates according to the protocol of the supplier. The ratio of firefly RLU to renilla RLU of each sample was automatically computed. The activity of each construct was expressed at the relative value compared to that of −525G allele, and these relative values were used for statistical analysis. Experiments were repeated three times in all cell lines.

Statistical analysis

Statistical analysis of association studies was performed using SNPAlyse software (DYNACOM, Yokohama, Japan). The presence of a Hardy–Weinberg equilibrium was examined using the χ^2 test for goodness of fit. Allele distributions between patients and comparison subjects were analyzed by the χ^2 test for independence. The measures of linkage disequilibrium (LD), denoted as D', was calculated from the haplotype frequency using an Expectation-Maximization algorithm. Case-control haplotype analysis was performed by the permutation method to obtain empirical significance [7]. Haplotype frequencies were determined by using the case-control haplotype analysis program of SNPAlyse software which was used in our previous study [15]. The global p values represent the overall significance using the χ^2 test when the observed vs expected frequencies of all the haplotypes are considered together. The individual haplotypes were tested for association by grouping all others together and applying the χ^2 test with 1 *df*. *P* values were calculated based on 10,000 replications. For luciferase reporter gene assay, Student's t test was used for comparison in relative luciferase expression between alleles inserted into vectors. All p values reported are two-tailed. Statistical significance was defined at $p<0.05$.

Results

The genotype distributions of all examined SNPs for the patients and controls were not significantly deviated from the Hardy–Weinberg equilibrium (data not shown). When we examined males and females separately, the genotype distributions of all examined SNPs for the male and female patients and controls were in the Hardy–Weinberg equilibrium (data not shown). Genotype distributions and allele

Table 1 Genotype and allele distributions for the SNPs in the GMIP gene among the patients with major depressive disorder and controls

SNP-ID	SNP	Controls				Major depressive disorder				OR (95% CI)
		Genotype frequency			Allele 2 frequency	Genotype frequency			Allele 2 frequency	
		1/1	1/2	2/2		1/1	1/2	2/2		
SNP1	G/A	134 (81.7%)	30 (18.3%)	0 (0%)	0.091	120 (73.2%)	41 (25.0%)	3 (1.8%)	0.143	1.66 (1.05–2.69)
SNP2	T/G	119 (72.6%)	44 (26.8%)	1 (0.6%)	0.140	106 (64.6%)	53 (32.3%)	5 (3.1%)	0.192	1.46 (0.93–2.26)
SNP3	G/C	120 (73.2%)	43 (26.2%)	1 (0.6%)	0.137	106 (64.6%)	53 (32.3%)	5 (3.1%)	0.192	1.50 (0.96–2.27)
SNP4	C/G	119 (72.6%)	44 (26.8%)	1 (0.6%)	0.140	106 (64.6%)	53 (32.3%)	5 (3.1%)	0.192	1.46 (0.96–2.23)

The major allele in each SNP represents allele 1

frequencies of the four SNPs among the patients and controls are shown in Table 1. The A allele of SNP1 was in excess in our MDD patients when compared to controls (χ^2=4.25, *df*=1, *p*=0.039), and there were trends toward increased frequencies of minor alleles of the other three SNPs (SNP2 *p*=0.075, SNP3 *p*=0.058, and SNP4 *p*=0.075). There was no difference in the allele frequency of SNP5 (8.7 kb away from the 5′ of the GMIP gene) or SNP6 (5.5 kb away from the 3′ of the GMIP gene) between patients and controls (*p*>0.2). As the association was strongest in the SNP1 in the putative promoter region, we have searched polymorphisms in the 5′-putative promoter region. We sequenced 1,280 bp of the 5′-upstream region of the GMIP gene using 24 unrelated Japanese subjects and detected one novel SNP (−1,007C/T). We genotyped this SNP by a PCR-based restriction-fragment-length polymorphism assay and found that the frequency of the minor allele (−1,007T) was rare (<1%), suggesting that this SNP has no major role in the pathogenesis of MDD.

As gender differences occur in MDD (female predominance), we examined males and females separately. Genotype distributions and allele frequencies of the four SNPs among the patients and controls in males (Table 2) and females (Table 3) are shown. There were significant differences in the allele frequencies for three SNPs between patients with MDD and controls in males (SNP2 *p*=0.0086, SNP3 *p*=0.0086, and SNP4 *p*=0.015), while there were no such differences for any SNPs between those with major depression and controls in females. Minor allele frequencies of SNP1 were overrepresented in both male and female patients with MDD, although the differences of the allele frequency did not reach statistical significance.

To further analyze the haplotype structure in our controls and MDD patients, we computed the LD between the SNPs using *D′* (Table 4). All *D′* values were more than 0.97, indicating tight LD across all the markers. Thus, we performed haplotype-based analysis consisting of the four SNPs. It was estimated that only two haplotypes represented more than 90% of the total haplotype diversity present in the population of total subjects. Estimated haplotype frequencies and individual *p* values corresponding to the haplotypes in patients with MDD and controls in males are shown in Table 5. The overall distribution of haplotypes was significantly different between MDD and controls in males (global *p* value=0.0087), while no significant difference was observed in females. The estimated frequency of the GTGC haplotype was significantly less in patients with MDD (86.4 vs 72.8%, *p*=0.0044) in males, while similar estimated frequencies of the GTGC haplotype were observed in females (MDD 84.8 vs control 85.2%). Our findings suggested that genetic variances in the genomic region of GMIP might be associated with MDD.

As −525G/A SNP in the putative promoter region had a significant association with MDD, the promoter analysis was carried out using luciferase reporter gene assay. To determine the effects of the −525G/A allele on transcription regulation, we cloned allele-specific promoter fragments into the pGL3-Basic vector (Promega). Promoter activity was assayed using a dual-luciferase system (Promega). Figure 2 shows observed relative luciferase expression

Table 2 Genotype and allele distributions for the SNPs in the GMIP gene among the patients with major depressive disorder and controls in male

SNP-ID	SNP	Controls				Major depressive disorder				OR (95% CI)
		Genotype frequency			Allele 2 frequency	Genotype frequency			Allele 2 frequency	
		1/1	1/2	2/2		1/1	1/2	2/2		
SNP1	G/A	45 (76.3%)	14 (23.7%)	0 (0%)	0.119	39 (66.1%)	17 (28.8%)	3 (5.1%)	0.195	180 (0.91–4.02)
SNP2	T/G	44 (74.6%)	15 (25.4%)	0 (0%)	0.127	32 (54.2%)	23 (39.0%)	4 (6.8%)	0.263	2.45 (1.23–5.23)
SNP3	G/C	44 (74.6%)	15 (25.4%)	0 (0%)	0.127	32 (54.2%)	23 (39.0%)	4 (6.8%)	0.263	2.45 (1.23–5.23)
SNP4	C/G	43 (72.9%)	16 (27.1%)	0 (0%)	0.136	32 (54.2%)	23 (39.0%)	4 (6.8%)	0.263	2.27 (1.20–4.72)

The major allele in each SNP represents allele 1

Table 3 Genotype and allele distributions for the SNPs in the GMIP gene among the patients with major depressive disorder and controls in females

SNP-ID	SNP	Controls				Major depressive disorder				OR (95% CI)
		Genotype frequency			Allele 2 frequency	Genotype frequency			Allele 2 frequency	
		1/1	1/2	2/2		1/1	1/2	2/2		
SNP1	G/A	89 (84.8%)	16 (15.2%)	0 (0%)	0.076	81 (77.1%)	24 (22.9%)	0 (5.1%)	0.114	1.56 (0.84–3.39)
SNP2	T/G	75 (71.4%)	29 (27.6%)	1 (1.0%)	0.148	74 (70.5%)	30 (28.6%)	1 (1.0%)	0.152	1.04 (0.61–1.80)
SNP3	G/C	76 (72.4%)	28 (26.7%)	1 (1.0%)	0.143	74 (70.5%)	30 (28.6%)	1 (1.0%)	0.152	1.08 (0.61–1.85)
SNP4	C/G	76 (72.4%)	28 (26.7%)	1 (1.0%)	0.143	74 (70.5%)	30 (28.6%)	1 (1.0%)	0.152	1.08 (0.61–1.85)

The major allele in each SNP represents allele 1

levels (RLEs) for the G or A alleles of the SNP1 of the GMIP gene compared to RLE without insertion of such alleles (empty pGL3-Basic vector). For all the cell lines examined (HeLa, IMR-32, and Hs683), RLE was robustly increased ($p<0.001$) due to insertion of the putative promoter region for both alleles compared to the empty pGL3-Basic vector. The RLE of the A allele, which was a higher allele frequency in patients, was significantly lower than that of the G allele in all cell lines (0.85±0.05 vs 1.00±0.03, $p<0.001$ in HeLa cells; 0.84±0.10 vs 1.00±0.07, $p=0.002$ in IMR-32 cells; 0.86±0.09 vs 1.00±0.07, $p=0.002$ in Hs683 cells, mean±SD). These results suggested that the putative promoter region, including −525G/A SNP, had a transcriptional activity, and that the difference in the transcription activity between the −525G and −525A alleles might influence the expression level of the GMIP gene.

Discussion

We found a significant association between genetic variations of the GMIP gene and MDD in a Japanese population. This significant association was observed among male subjects in particular. Men are about half as likely as women to experience a lifetime episode of major depression [5]. The exact etiology of this gender difference is unclear; however, psychological factors, changes in circulating gonadal hormones, and neurological factors are likely to contribute. Our results, stronger evidence for association in men, can lead to one of neurological factors, as the GMIP gene is a member of Rho GTPase-activating proteins which play a crucial role in neuronal development and synaptic functions [13]. Impairment of these regulations may alter the neural circuit in the central nervous system. Differences in brain development between the sexes arise from the differential actions of genes that are encoded on the sex chromosomes [2]. Actually, several genes of X-linked mental retardation, which occurs in males, are involved in Rho signaling pathways such as oligophrenin 1, PAK3, etc. [18]. Interestingly, a Rho GTPase-activating protein, the chimerin 2 gene, is reported to be associated with schizophrenia in men [8]. Taken together, Rho signaling genes can confer the susceptibility for mental disorders particularly in men.

Table 4 Marker-to-marker LD in the GMIP gene

	SNP1	SNP2	SNP3	SNP4
SNP1	–	0.97	0.97	0.97
SNP2	1.00	–	1.00	1.00
SNP3	1.00	1.00	–	1.00
SNP4	1.00	0.97	1.00	–

For each pair of markers, the standardized *D'* in controls is shown below the diagonal, and the standardized *D'*, in cases above the diagonal.

Table 5 Estimated haplotype frequencies for patients and controls in males

Haplotype	SNP1	SNP2	SNP3	SNP4	Haplotype frequency		P value
					Patients	Controls	
1	G	T	G	C	0.728	0.864	0.0044
2	A	G	C	G	0.186	0.119	0.11

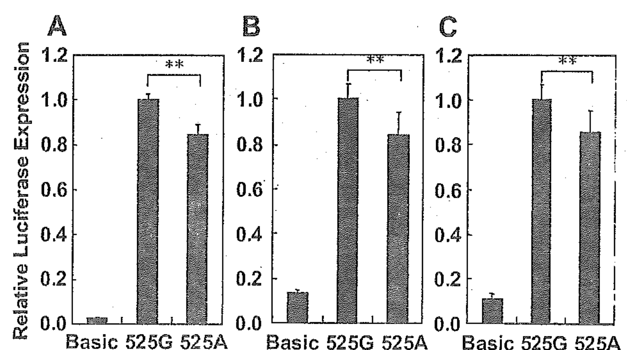

Fig. 2 Effects of human GMIP −525G/A SNP on promoter activity measured using HeLa cells (**a**), IMR-32 cells (**b**), and Hs683 cells (**c**). The figures show firefly luciferase activity relative to Renilla luciferase activity derived from the internal control vector phRL-TK. The construct containing the −525G allele was assigned a value of *1*. The pGL3-Basic vector (*Basic*), which does not contain promoter sequence, was used as a negative control. Results are means± SD of three independent transfection experiments performed in triplicate. ** indicates $p<0.005$

The four SNPs in the genomic region of GMIP were in strong LD in both MDD patients and controls. There were two common haplotypes (allele frequency in controls GTGC 0.86 and AGCG 0.12). When we computed LD between six SNPs, including two SNPs located outside of the GMIP gene, we also found strong LD (all D'>0.96). An unknown functional polymorphism, which is in LD with the associated SNPs and/or haplotypes in the GMIP gene and/or nearby genes, may be responsible for giving susceptibility for MDD.

Among all examined SNPs, the strongest evidence for association with MDD was observed for the −525G/A SNP. We calculated the power to detect a significant difference using our sample sizes for the −525G/A SNP in total samples (164), as well as males (59) or females (105), with a power of 90% when the critical p value was set at 0.05. The minor allele frequencies for the −525G/A SNP in controls were 0.091 (total), 0.119 (male), and 0.076 (female). The required odds ratios to detect a significant association between the risk allele and MDD were 1.7 (total), 3.0 (male), and 2.7 (female), while empirical odds ratios were 1.7 (total), 1.8 (male), and 1.6 (female). Thus, the reason why we did not detect the significant difference in males and females might be due to the power, which is not high enough to detect it. To detect the significant difference with a power of 90% when the critical p value was set at 0.05 in males (allele frequency 0.119, OR 1.8) and females (allele frequency 0.076, OR 1.6), sample sizes of 236 (males) and 561 (females) are required. The sample size we used did not have sufficient power to detect an interaction between the effect of the gene and gender on the disease. However, our results within the constraints of limited power indicate that the −525G/A SNP might be associated with MDD. Further studies to confirm the association between the SNP and MDD are warranted in such a larger sample size.

Since −525G/A SNP is located in the putative promoter region, it may play a role in transcriptional regulation. Luciferase reporter assay revealed the transcriptional activity of the DNA fragment of the 5′-upstream region of the GMIP gene, including −525G/A SNP. These findings were observed in three types of human cell lines (HeLa, IMR-32, and Hs683) where GMIP mRNA has been confirmed by reverse transcription–PCR (data not shown). Indeed, the −525A allele fragment, which was a higher allele frequency in MDD, showed lower promoter activity than the −525G allele fragment. The G to A nucleotide transition can alter the consensus sequence of binding sites of several transcription factors, including ZID (zinc finger protein with interaction domain), CP2, FTF (fetoprotein transcription factor), and MEF-3 [3, 10, 16, 17]. The G to A nucleotide transition might lose the binding activity on CP2 or ZID and might generate the binding activity on FTF or MEF-3. ZID binding activity was predicted to be most affected by motif sequences among the four transcription factors. Ribonuclease (Rnase) protection analysis indicated a wide expression of ZID, with highest levels in the brain, suggesting that ZID binding activity might be altered by the promoter SNP which was associated with MDD. Our polymorphism screening of the 5′-upstream region of the GMIP gene identified only one additional rare SNP (−1,007C/T). We searched a missense SNP in the databases (NCBI, JSNP, and Celera) and found only one validated missense SNP of the GMIP gene (rs12003; D641N). However, the minor allele of this SNP was not detected in our 328 Japanese samples, while the frequency of the minor allele in a Caucasian is 0.06. Further investigation is necessary to find a functional polymorphism which can be responsible for the susceptibility of MDD.

In conclusion, we have demonstrated for the first time the possible association between the genetic variation of the GMIP gene and MDD in a Japanese population. A false-positive association due to population stratification, and possible confounding factors such as stressful life events, adverse childhood experiences, and certain personality traits, socioeconomic status, etc., cannot be excluded in our case-control design, despite the precaution of ethnic matching of this study. Therefore, it is necessary to carry out further investigations to confirm our findings in other samples. If our results are replicated, potential −525G/A SNP function on transcriptional activity might contribute to understanding the molecular mechanisms of MDD.

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