An association study of tachykinin receptor 3 gene with schizophrenia in the Japanese population

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The tachykinin receptor 3 (*TACR3*) gene encodes the neurokinin3 (NK3) receptor. Animal studies showed that agonist-induced stimulation of the NK3 receptor leads to the excessive release of dopamine in the ventral and dorsal striatal and prefrontal cortical regions. Data from clinical trials of selective NK3 receptor antagonists in schizophrenia have shown significant improvement in positive symptoms. We performed an association study of the *TACR3* gene in the Japanese population of 384 schizophrenic patients and 384 controls. Nine single nucleotide polymorphisms were genotyped using TaqMan assays and polymerase chain reaction-restriction fragment length polymorphism method. No significant association between schizophrenia and these single nucleotide polymorphisms was observed in single-marker and haplotype analyses. Our results suggest that *TACR3* is unlikely to be related to the development of schizophrenia in the Japanese population. *NeuroReport* 19:471–473 © 2008 Wolters Kluwer Health | Lippincott Williams & Wilkins.

Keywords: association study, dopamine, neurokinin3 receptor, schizophrenia, tachykinin receptor 3

Introduction

Schizophrenia is a severe, disabling and lifelong mental disorder with a global prevalence of 1%. Although it is generally accepted that genetic factors contribute to the development of this disease, its etiology has not yet been clarified.

The tachykinin receptor 3 (*TACR3*) gene located on chromosome 4q25 encodes the neurokinin 3 receptor (NK3). Its endogenous ligand is neurokinin B, a member of the tachykinin peptide family. It is expressed in the central nervous system and spinal cord [1]. Several animal studies have been performed investigating the interaction between NK3 receptors and dopamine (DA) and serotonin (5-HT) pathways. First, stimulation of NK3 receptors in the ventral mesencephalon increases DA release in the ventral and dorsal striatum and prefrontal cortex [2,3]. Second, infusion of NK3 receptor agonists into the ventral tegmental area evokes DA-mediated behaviors, such as yawning and chewing, which are potently inhibited by DA2 receptor antagonists such as haloperidol [4,5]. Third, injection of NK3 receptor agonists into the raphe area elicits 5-HT-mediated behaviors, such as head twitches, which are inhibited by 5-HT2A/5-HT2C receptor antagonists [6,7]. Additionally, a recent clinical study showed NK3 receptor antagonists improved the positive symptoms in schizophrenia [8]. These results suggest that altered form and function of the NK3 receptor might be related to the abnormalities of DA and 5-HT signaling, one of the major hypotheses explaining the pathophysiology of schizophrenia.

For all of these reasons, *TACR3* was hypothesized to be involved in the pathogenesis of schizophrenia. In this study, we performed linkage disequilibrium (LD) analysis of the *TACR3* gene and carried out case–control association studies between TACR3 polymorphisms and schizophrenia using single-marker association analysis and haplotype analysis in the Japanese population.

Methods

Participants

A total of 384 patients with schizophrenia [231 men, mean age ± standard deviation (SD) 48.8 ± 14.4; 153 women, 53.0 ± 15.9] and 384 controls (161 men, 40.8 ± 15.6; 223 women, 39.5 ± 13.8) were genotyped. All participants were ethnically Japanese and unrelated to each other. The schizophrenic patients, who were diagnosed according to the DSM-IV criteria with the consensus of at least two experienced psychiatrists on the basis of empirical diagnostic interviews and review of medical records, were recruited from several psychiatric hospitals around the Tokyo and Nagoya areas (within a 350 km radius). All healthy control participants with no current or past contact with psychiatric services were also screened on the basis of brief diagnostic interviews. This study was approved by the Ethics Committee of the Nagoya University Graduate School of Medicine, and written informed consent was obtained from each participant.

Haplotype tag single nucleotide polymorphism selection

We first consulted the HapMap database (release #16c.1, *www.hapmap.org*) and determined the LD block with the criteria $D' > 0.8$ using HAPLOVIEW ver. 3.2 software [9]. All single nucleotide polymorphisms (SNPs) listed in the entire coding region as well as the 500 bp upstream 5′-flanking region and 500 bp downstream 3′-UTR region (minor allele frequency >0.05) were included in the LD analysis. Haplotype tag SNPs (htSNPs) were defined as those capturing 90% of the haplotype diversity within each LD block using the same program. The Japanese portion of the HapMap data was used for this procedure (Fig. 1).

Single nucleotide polymorphism genotyping

Genotyping of the htSNPs was carried out using TaqMan assays (Applied Biosystems, Foster City, California, USA) and the PCR-restriction fragment length polymorphism (RFLP) method (Table 1). TaqMan probes and Universal PCR Master Mix were obtained from Applied Biosystems. A 5-μl total reaction volume was used, and allelic-specific fluorescence was measured using the ABI PRISM 7900 Sequence Detector System (Applied Biosystems). Detailed information on the PCR method is available upon request.

Statistical analysis

Genotype deviation from the Hardy–Weinberg equilibrium (HWE) was evaluated by the χ^2 test. Single marker association and haplotype analyses were performed with SPSS version 11.0J (Tokyo, Japan) and COCAPHASE version 2.403 (*http://portal.litbio.org/Registered/Option/unphased*; Dudbridge, 2003), respectively. The significance level for all statistical tests was 0.05. Power calculations were performed using the genetic statistical package Genetic Power Calculator (*http://pngu.mgh.harvard.edu/~purcell/gpc/*; Purcell 2001–2005).

Results

The *TACR3* gene was composed of six LD blocks. One nonblock SNP and eight htSNPs were finally selected according to the criteria (Fig. 1). The genotype and allele frequency of each htSNP in schizophrenic patients and controls are summarized in Table 1. The observed genotype frequencies of all SNPs were within the distribution expected according to HWE. Neither the genotype nor allele frequency of any SNP differed significantly between the schizophrenia group and the control group (Table 1). The distribution of haplotype frequencies did not differ significantly between the schizophrenic patients and controls (Table 2). More than 80% power in detecting any association with schizophrenia was obtained when the genotype relative risk was set at 1.33–1.66 under a multiplicative model of inheritance.

Discussion

Our study indicates that the *TACR3* gene does not play a major role in the development of schizophrenia in the Japanese population, as no significant differences in allele, genotype, or haplotype frequencies of the selected SNPs were found between schizophrenic patients and controls. As it is, however, suspected that genetic risk factors for schizophrenia may differ between races or ethnicities, a replication study including different ethnic populations is needed to validate these results.

As mentioned in the Introduction, the NK3 receptor was reported to regulate the DA and 5-HT release or concentration at the synapse. It would therefore be valuable to investigate the gene–gene interactions between *TACR3* and other DA or 5-HT signaling related genes [10]. Furthermore, the 5-HT1A receptor partial agonist tandospirone is reported to be effective as an adjunctive treatment to improve cognition in patients with schizophrenia [11]. As the NK3 receptor is thought to have the potential for indirect influence on the 5-HT1A receptor through 5-HT release, association analysis using samples with data on the cognitive function might help elucidate the pathogenesis of schizophrenia.

A couple of limitations in this study should be considered. First, the male/female ratios and average ages did not match between schizophrenic patients and controls. When we performed a multiple regression analysis, there were no effects of age or sex on the disease status (data not shown). Additionally, these effects might be small because not likely

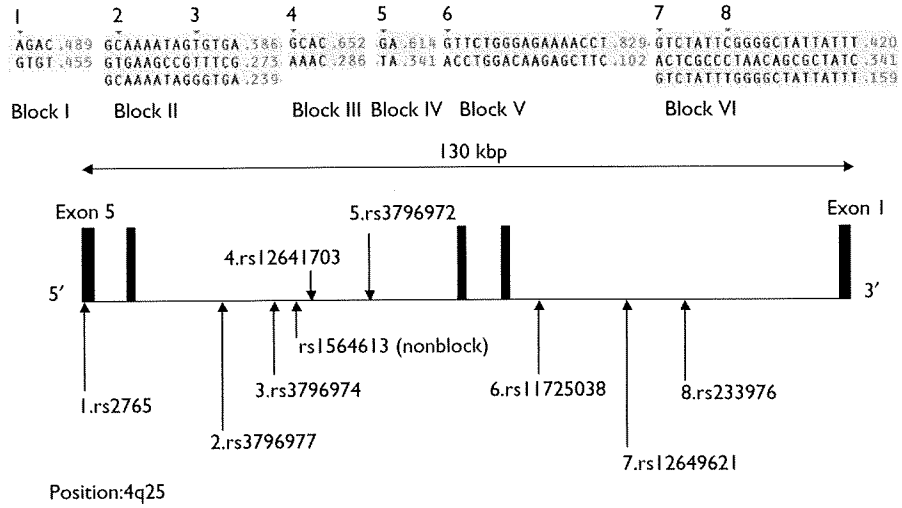

Fig. 1 Genomic structure of *TACR3* with haplotype tag single nucleotide polymorphisms (SNPs) and haplotype frequencies in each linkage disequilibrium block provided by HapMap database V.16. Numbers under or above the arrows represent the SNPs we selected in this study.

Table 1 Association analyses of haplotype tag SNPs

SNP	Block	Method of genotyping		GRR	Allelic distribution[a]			Genotypic distribution[a]			
					M	m	*P* value	M/M	M/m	m/m	*P* value
rs2765	I	TaqMan	SCZ	1.33	411	345	0.685	114	183	81	0.861
			CONT		401	351		112	178	87	
rs3796977	II	TaqMan	SCZ	1.41	611	153	0.471	249	113	20	0.79
			CONT		598	164		240	120	22	
rs3796974	II	TaqMan	SCZ	1.34	502	264	0.992	165	172	46	0.563
			CONT		493	259		169	156	52	
rs12641703	III	TaqMan	SCZ	1.35	506	260	0.669	167	172	44	0.536
			CONT		485	261		164	157	52	
rs3796972	IV	PCR-RFLP	SCZ	1.33	406	350	0.45	106	195	78	0.397
			CONT		414	330		121	177	77	
rs11725038	V	TaqMan	SCZ	1.37	556	210	0.899	204	149	31	0.954
			CONT		548	210		201	146	33	
rs12649621	VI	TaqMan	SCZ	1.33	430	336	0.284	127	178	79	0.53
			CONT		407	355		113	183	87	
rs233976	VI	TaqMan	SCZ	1.44	639	123	0.833	270	100	12	0.614
			CONT		642	120		269	106	8	
rs1564613	Non-block	TaqMan	SCZ	1.66	710	52	0.799	331	48	2	0.793
			CONT		705	49		332	43	3	

CONT, control; GRR, genotype relative risk; M, major allele; m, minor allele; SCZ, schizophrenia; SNP, single nucleotide polymorphism.
[a]In absolute numbers.

Table 2 Haplotype analyses

Block	SNP	Haplo-type	SCZ[a]	CON[a]	*P* value[b]	Global *P* value[b]
		AT	0.455	0.44	0.558	
2	rs3796977-rs3796974	AC	0.345	0.344	0.993	0.734
		CT	0.2	0.215	0.467	
		GG	0.439	0.466	0.284	
6	rs12649621-rs233976	AG	0.4	0.377	0.348	0.531
		AA	0.161	0.157	0.837	

CONT, control; SCZ, schizophrenia; SNP, single nucleotide polymorphism.
[a]Estimated frequencies.
[b]*P* values were calculated by log-likelihood ratio test.

more than four participants given a lifetime morbidity risk of 1% will eventually develop schizophrenia. Second, we selected htSNPs so as to cover 90% of the haplotypes within each LD block. It is, however, possible that the htSNPs used in this study did not capture all haplotypes in the gene, as the LD block structure of *TACR3* was not tight. In other words, there may be SNPs not found in the LD, for which we did not investigate the possible association with schizophrenia. Thus, further analysis based on more comprehensive and detailed SNP coverage of *TACR3* is required to make conclusive results.

Conclusion

The present results suggest that *TACR3* itself is unlikely to be related to the development of schizophrenia in the Japanese population. Further studies including pharmacogenetic investigations are required, however, for conclusive results on the exact roles of *TACR3* in the pathophysiology of schizophrenia.

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A polymorphism of the metabotropic glutamate receptor mGluR7 (*GRM7*) gene is associated with schizophrenia

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Abstract

Introduction: Glutamate dysfunction has been implicated in the pathophysiology of schizophrenia. The metabotropic glutamate receptors (mGluRs) are G-protein-coupled receptors. *GRM7*, the gene that encodes mGluR7, is expressed in many regions of the human central nervous system. The *GRM7* gene is located on human chromosome 3p26, which has been suggested by linkage analysis to contain a susceptibility locus for schizophrenia.
Methods: We screened for mutations in all exons, exon/intron junctions, and promoter regions of the *GRM7* gene in Japanese patients with schizophrenia and evaluated associations between the detected polymorphisms and schizophrenia. We examined the influence of one polymorphism associated with schizophrenia on the expression of *GRM7* by dual-luciferase assay in transfected cells.
Results: Twenty-five polymorphisms/mutations were detected in *GRM7*. Case-control analysis revealed a potential association of a synonymous polymorphism (371T/C, rs3749380) in exon 1 with schizophrenia in our case-control study of 2293 Japanese patients with schizophrenia and 2382 Japanese control subjects (allelic $p=0.009$). Dual-luciferase assay revealed suppression of

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transcription activity by exon 1 containing this polymorphism and a statistically significant difference in the promoter activity between the T and C alleles.
Conclusions: Our results support the possible association of a *GRM7* gene polymorphism with genetic susceptibility to schizophrenia.

Keywords: Glutamate; Expression; Luciferase; Prefrontal

1. Introduction

Schizophrenia is a severe psychiatric disorder, which is equally prevalent in men and women and affects approximately 1% of the population worldwide. Several neurotransmitter systems and functional networks within the brain have been found to be affected in patients with schizophrenia. The glutamatergic neuronal dysfunction hypothesis is one of the main explanatory hypotheses (Carlsson et al., 1997). Glutamate is the primary excitatory neurotransmitter in brain. It is contained as a neurotransmitter in approximately 60% of brain neurons, including almost all cortical pyramidal neurons. Further, virtually 100% of brain neurons contain some type of glutamate receptor. Glutamate mediates its effects on the central nervous system via both ionotropic and metabotropic receptors. The metabotropic glutamate receptors (mGluRs), which are G-protein-coupled receptors, are divided into 3 groups on the basis of sequence homology, putative signal transduction mechanisms, and pharmacologic properties (Nakanishi, 1994; Pin and Duvoisin, 1995). The mGluRs in group I are mGluR1 and mGluR5, those in group II are mGluR2 and mGluR3, and those in group III are mGluRs 4, 6, 7, and 8. Group II and group III mGluRs are linked to inhibition of the cyclic AMP cascade but differ in their agonist selectivities.

mGluR7 is the most highly conserved mGluR subtype across mammalian species (Flor et al., 1997). Makoff et al. (1996) observed by in situ hybridization that *GRM7* is expressed in many areas of the human brain, especially the cerebral cortex, hippocampus, and cerebellum. mGluR7 is localized directly in the presynaptic zone of the synaptic cleft of glutamatergic synapses (Kinoshita et al., 1998; Kosinski et al., 1999), where it is thought to act as an autoreceptor that is activated by glutamate released from the presynaptic terminal during action potentials. Furthermore, mGluR7 is thought to be a key player in shaping synaptic responses at glutamatergic synapses as well as in regulating key aspects of inhibitory GABAergic transmission (Kinoshita et al., 1998; Kosinski et al., 1999).

mGluR7 has putative roles in anxiety, emotional responses, and spatial working memory (Callaerts-Vegh et al., 2006; Cryan et al., 2003; Mitsukawa et al., 2006). Cognitive dysfunction is estimated to occur in 75%–85% of patients with schizophrenia, often precedes the onset of other symptoms (Reichenberg et al., 2006). Working memory is one of primary cognitive domains that are crucial for developing targets for the treatment of cognition in schizophrenia (Nuechterlein et al., 2004).

mGluR7 ablation causes dysregulation of the hypothalamic–pituitary–adrenal axis and increases hippocampal BDNF protein levels (Mitsukawa et al., 2005). Dysregulation of BDNF production or release is associated with neuropsychiatric disorders, such as schizophrenia (Harrison and Weinberger, 2005). Association between the val66met polymorphism of the *BDNF* gene and hippocampal volume in human, particularly in patients with schizophrenia (Szeszko et al., 2005).

In the present study, we examined the *GRM7* gene as a candidate for schizophrenia.

2. Materials and methods

2.1. Subjects

All subjects were of Japanese descent and were recruited from the main island of Japan. Patients with schizophrenia were diagnosed according to the Diagnostic and Statistical Manual of Mental Disorders, Fourth Edition (DSM-IV). Control subjects were mentally healthy, unrelated subjects with no self-reported family history of mental illness within second-degree relatives. We sequenced the 5′ region, exons and exon–intron boundaries of the *GRM7* gene in 32 patients (mutation screening patients) with schizophrenia (mean age±SD, 46.5±10.9 years; 17 men and 15 women) to identify polymorphisms. We then genotyped 576 patients including the mutation screening patients (mean age±SD, 46.6±14.8 years; 322 men and 254 women) and 576 control subjects (mean age±SD, 46.8±12.9 years; 268 men and 308 women) (1st association population) with Predesigned TaqMan single nucleotide polymorphism (SNP) genotyping assays. When Predesigned TaqMan SNP genotyping assays were not available, we performed direct sequencing of DNAs from 96 patients including the mutation screening

patients (mean age±SD, 50.3±13.1 years; 55 men and 40 women) and 96 control subjects (mean age±SD, 53.6±9.1 years; 42 men and 54 women). For SNPs for which an association with schizophrenia was suggested in the first association population, we performed genotyping in an independent sample of 1817 patients (mean age± SD, 45.5±14.1 years; 962 men and 855 women) and 1728 control subjects (mean age±SD, 46.2±13.6 years; 958 men and 770 women) (confirmation population). The present study was approved by the ethics committees of the University of Tsukuba and participating institutes. All participants provided written informed consent.

2.2. DNA isolation and genotyping

DNAs were extracted from peripheral lymphocytes by standard phenol–chloroform extraction. The genomic structure of *GRM7* was determined from the University of California at Santa Cruz (UCSC) database (http://genome.ucsc.edu/cgi-bin/hgGateway) and the National Center for Biotechnology Information (NCBI) database (http://www.ncbi.nlm.nih.gov/). To screen for polymorphisms, we performed direct sequencing of genomic DNAs with a Big Dye Terminator Cycle Sequencing Kit and the ABI PRISM 3100 Genetic Analyzer (Applied Biosystems, Foster City, CA, USA). All exons, exon–intron junctions, and 1.6 kb of the 5′ flanking region of *GRM7* were amplified from the genomic DNAs of 24 randomly selected patients. The sequences of primers and conditions used for amplification for the mutation screening are available upon request. We genotyped polymorphisms with the TaqMan SNP Genotyping Assay (Applied Biosystems) and ABI PRISM 7900HT Sequence Detection System (Applied Biosystems).

2.3. Real-time quantitative reverse transcription-polymerase chain reaction (RT-PCR)

Total RNA was isolated from cultured cells with the SV Total RNA Isolation System (Promega, Madison, WI, USA). cDNA was synthesized from RNA with Revertra Ace (Toyobo, Tokyo, Japan) and oligo dT primer. Expression of *GRM7* was quantified by real-time quantitative RT-PCR with the TaqMan Gene Expression Assay and ABI PRISM 7900HT Sequence Detection System (Applied Biosystems) per the manufacturer's instructions. Primers and probes were purchased from Applied Biosystems (Assays-on-Demand Assay ID: Hs00179051_m1). *GAPDH* was used as an internal control. Data were collected and analyzed with Sequence Detector Software (SDS) version 2.1 (Applied Biosystems) and the standard curve method.

2.4. Luciferase reporter assay

To assay promoter activity of the 5′-flanking region and exon 1 of the *GRM7* gene, 9 fragments of the 5′ region were cloned into the pGL3-Basic plasmid with and without a Simian virus 40 enhancer sequence (Promega, Madison, WI, USA). The day before transfection, NH-12 cells (Japanese Collection of Research Bioresources Gene Bank, http://genebank.nibio.go.jp/gbank/index_e.html) were plated at 1×10^5 cells/well in a 24-well plate and grown in DMEM (Sigma, St. Louis, MO, USA) supplemented with 10% fetal bovine serum (FBS) (Equitech-Bio, Kerrville, TX, USA). One microgram of each test plasmid was transiently cotransfected into the cells with 0.1 μg of pRL-TK plasmid (an internal standard reporter) (Promega) with Lipofectamine 2000 (Invitrogen) per manufacturer's protocol. After 48 h, the dual-luciferase assay was performed with a PicaGene Dual SeaPansy Kit (Toyo Ink, Tokyo, Japan) according to the manufacturer's instructions.

2.5. Statistics

Deviation from predicted Hardy–Weinberg frequencies was examined by chi-square test. Individual allelic associations were examined by Fisher's exact test. Genotypic associations were examined by Armitage's Trend Test for the reasons discussed by Devlin and Roeder (1999). A significant association was defined when the given p value for allelic or genotypic tests was less than 5% (uncorrected $p<0.05$) and the same association was confirmed in an independent population with $p<0.05$. Linkage disequilibrium (LD) between polymorphisms and haplotype block structures was evaluated with Haploview software version 3.11 (Barrett et al., 2005). Haplotype blocks were generated with the default algorithm taken from Gabriel et al. (2002). Haplotypic associations with disorders were examined with Haploview software, which performs association tests on the set of blocks selected by obtaining counts for case-control association tests by summing the fractional likelihoods of each individual for each haplotype by the EM algorithm.

3. Results

Twenty-five polymorphisms/mutations were detected in the exons, exon–intron junctions, and 5′-flanking region of the *GRM7* gene (Fig. 1). Genotyping was carried out for all detected polymorphisms except rare variants with allele frequencies<0.05 and polymorphisms in LD with each other ($r^2=1$). Among these SNPs, 1724A/G (rs34373930), 1938C/T (rs7614915), IVS8+

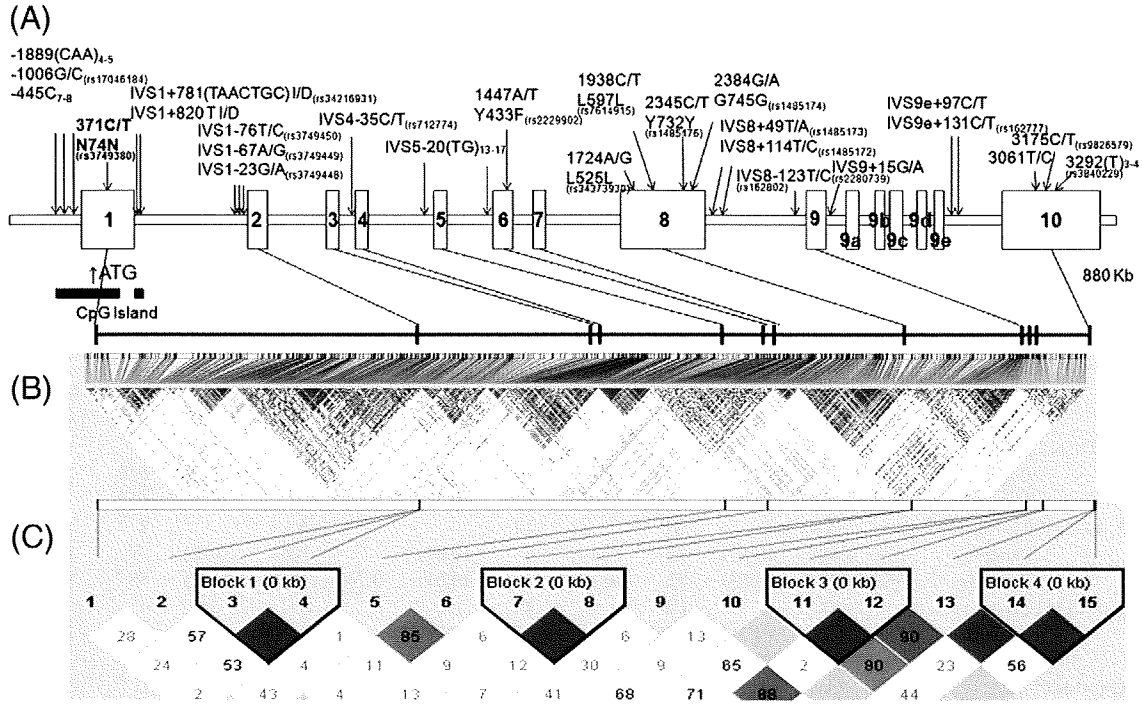

Fig. 1. Schematic representation of the *GRM7* gene and relevant mutations/polymorphisms (A), linkage disequilibrium plot of the Japanese population from HapMap database (B), and between SNPs genotyped in this study shown in Table 1 (C). Figures in rhombus are D' between SNPs and filled rhombus without figures indicates $D'=1$ in (C).

49T/A (T/A at the position of 49 base-pair starting from the G of the donor site of intron 8), and 2345C/T (rs1485175) were in complete LD ($r^2=1$), and IVS8+114T/C and 2384G/A (rs1485174) were also in complete LD ($r^2=1$) in 24 screening samples. Therefore, we genotyped the 2345C/T and 2384G/A polymorphisms as representative SNPs. The IVS9+97C/T, 2912T/C, and 3292(A)3-4 (rs3840229) SNPs were genotyped in 96 patients and 96 controls by direct sequencing, and the other 12 SNPs were genotyped in 576 patients and 575 control subjects by TaqMan SNP Genotyping Assay (Applied Biosystems). The genotypic distributions of these 15 SNPs did not deviate significantly from Hardy–Weinberg equilibrium ($p>0.05$). A synonymous polymorphism (371T/C) in exon 1 of *GRM7* showed a potential association for schizophrenia (allelic uncorrected $p=0.04$) (Table 1). We did not detect a significant association with schizophrenia of any of the other 14 SNPs, including the Tyr433Phe polymorphism (allelic uncorrected $p=0.33$; genotypic uncorrected $p=0.63$), which was previously reported to be associated (Bolonna et al., 2001). The haplotype blocks consisted of IVS1-76T/C, IVS1-67A/G, and IVS1-23G/A and of 2345C/T and 2384A/G. No significant haplotypic association was suggested for these 2 haplotype blocks ($p>0.05$). We confirmed the association of the 371T/C polymorphism with schizophrenia in an independent population of 1717 patients and 1807 control subjects and confirmed the association (allelic $p=0.03$, one-sided) (Table 2). The allelic p value of the association in the total population of 2293 patients with schizophrenia and 2382 control subjects was 0.009 (Table 2). According to the HapMap database (http://www.hapmap.org/index.html), the 371T/C polymorphism (rs3749380) was not in the LD block and was not in LD with other SNPs within 80 kb ($r^2<0.7$). Weak LD was observed with rs458351 ($D'=0.89$ and $r^2=0.69$), which is 30 kb upstream of rs3749380.

Expression of *GRM7* mRNA was assessed by RT-PCR in 7 different human cell lines (IMR-32, NH-12, TN-2, NB-1, SCCH-26, A172, and T98G). *GRM7* was expressed in NH-12 and SCCH-26 cells (data not shown). Because expression in NH-12 cells was higher than in SCCH-26 cells, we used NH-12 cells, a human cell line derived from neuroblastoma, for luciferase assays. Dual-luciferase assay revealed that the strongest promoter activity for *GRM7* was contained in the 1-kb fragment upstream of the ATG site of exon 1. However, a promoter construct containing the sequence from the ATG to the end of exon 1 showed significantly lower activity, indicating that the 371T/C polymorphism is in a regulatory region. When an SV40

Table 1
Genotypic and allelic distributions of polymorphisms in the *GRM7* gene

Polymorphism	Population	*n*	Genotype count (frequency)						*p*	Allele count (frequency)				*p*
371T/C (rs3749380)			CC		TC		TT			C		T		
	Patients	576	241	(0.42)	256	(0.44)	79	(0.14)		738	(0.64)	414	(0.36)	
	Controls	575	274	(0.48)	236	(0.41)	65	(0.11)	0.04	784	(0.68)	366	(0.32)	0.04
IVS1-76T/C (rs3749450)			CC		TC		TT			C		T		
	Patients	568	10	(0.02)	148	(0.26)	410	(0.72)		168	(0.15)	968	(0.85)	
	Controls	561	18	(0.03)	129	(0.23)	414	(0.74)	0.95	165	(0.15)	957	(0.85)	0.96
IVS1-67A/G (rs3749449)			GG		AG		AA			G		A		
	Patients	570	8	(0.01)	95	(0.17)	467	(0.82)		111	(0.10)	1029	(0.90)	
	Controls	567	7	(0.01)	89	(0.16)	471	(0.83)	0.60	103	(0.09)	1031	(0.91)	0.59
IVS1-23G/A (rs3749448)			AA		GA		GG			A		G		
	Patients	569	26	(0.05)	181	(0.32)	362	(0.64)		233	(0.20)	905	(0.80)	
	Controls	566	30	(0.05)	178	(0.31)	358	(0.63)	0.75	238	(0.21)	894	(0.79)	0.75
IVS4-35C/T (rs712774)			CC		CT		TT			C		T		
	Patients	571	121	(0.21)	260	(0.46)	190	(0.33)		502	(0.44)	640	(0.56)	
	Controls	559	117	(0.21)	268	(0.48)	174	(0.31)	0.66	502	(0.45)	616	(0.55)	0.65
1447T/A (rs2229902, Phe433Tyr)			AA		AT		TT			A		T		
	Patients	575	488	(0.85)	81	(0.14)	6	(0.01)		1057	(0.92)	93	(0.08)	
	Controls	569	484	(0.85)	82	(0.14)	3	(0.01)	0.76	1050	(0.92)	88	(0.08)	0.75
2345C/T (rs1485175)			CC		CT		TT			C		T		
	Patients	569	120	(0.21)	275	(0.48)	174	(0.31)		515	(0.45)	623	(0.55)	
	Controls	562	118	(0.21)	282	(0.50)	162	(0.29)	0.69	518	(0.46)	606	(0.54)	0.69
2384A/G (rs1485174)			AA		GA		GG			A		G		
	Patients	569	18	(0.03)	150	(0.26)	401	(0.70)		186	(0.16)	952	(0.84)	
	Controls	561	24	(0.04)	162	(0.29)	375	(0.67)	0.15	210	(0.19)	912	(0.81)	0.14
IVS8-123T/C (rs162802)			CC		TC		TT			C		T		
	Patients	569	3	(0.01)	91	(0.16)	475	(0.83)		97	(0.09)	1041	(0.91)	
	Controls	566	9	(0.02)	88	(0.16)	469	(0.83)	0.49	106	(0.09)	1026	(0.91)	0.48
IVS9+15G/A (rs2280739)			AA		GA		GG			A		G		
	Patients	570	4	(0.01)	84	(0.15)	482	(0.85)		92	(0.08)	1048	(0.92)	
	Controls	564	3	(0.01)	86	(0.15)	475	(0.84)	0.94	92	(0.08)	1036	(0.92)	0.94
IVS9e+97C/T			CC		CT		TT			C		T		
	Patients	96	75	(0.78)	20	(0.21)	1	(0.01)		170	(0.89)	22	(0.11)	
	Controls	95	78	(0.82)	16	(0.17)	1	(0.01)	0.53	172	(0.91)	18	(0.09)	0.62
IVS9e+131C/T (rs162777)			CC		CT		TT			C		T		
	Patients	570	22	(0.04)	186	(0.33)	362	(0.64)		230	(0.20)	910	(0.80)	
	Controls	566	23	(0.04)	178	(0.31)	365	(0.64)	0.82	224	(0.20)	908	(0.80)	0.82
2912T/C			TT		TC		CC			T		C		
	Patients	96	78	(0.81)	18	(0.19)	0	(0.00)		174	(0.91)	18	(0.09)	
	Controls	95	83	(0.87)	12	(0.13)	0	(0.00)	0.32	178	(0.94)	12	(0.06)	0.34
3175C/T (rs9826579)			CC		CT		TT			C		T		
	Patients	574	25	(0.04)	203	(0.35)	346	(0.60)		253	(0.22)	895	(0.78)	
	Controls	567	23	(0.04)	189	(0.33)	355	(0.63)	0.44	235	(0.21)	899	(0.79)	0.44
3292(A)3–4 (rs3840229)			33		34		44			3		4		
	Patients	96	80	(0.83)	16	(0.17)	0	(0.00)		176	(0.92)	16	(0.08)	
	Controls	95	78	(0.82)	17	(0.18)	0	(0.00)	0.85	173	(0.91)	17	(0.09)	0.86

enhancer was added downstream of the luciferase gene, the 371C allele showed significantly higher promoter activity than the 371T allele (Fig. 2).

4. Discussion

In the present study, we examined associations between polymorphisms in the *GRM7* gene and schizophrenia. Weak association was found for a synonymous SNP (371T/C) in exon 1 in the first association population, and this association was replicated in the confirmation population. The T allele, which is associated with schizophrenia, has lower promoter activity than the C allele. On the basis of this finding, we hypothesized that lower expression of mGluR7 may increase risk of developing schizophrenia, though studies of the expression of GRM7 in brains of patients with schizophrenia have not been reported.

Table 2
Association of the 371T/C polymorphism (rs3749380) in the *GRM7* gene with schizophrenia

Polymorphism population	*n*	Genotype count (frequency)						*p*	Allele count (frequency)				*p*
		CC		CT		TT			C		T		
Screening population													
Patients	576	241	(0.42)	256	(0.44)	79	(0.14)		738	(0.64)	414	(0.36)	
Controls	575	274	(0.48)	236	(0.41)	65	(0.11)	0.04	784	(0.68)	366	(0.32)	0.04
Confirmatory population													
Patients	1717	715	(0.42)	771	(0.45)	231	(0.13)		2201	(0.64)	1233	(0.36)	
Controls	1807	799	(0.44)	794	(0.44)	214	(0.12)	0.07	2392	(0.66)	1222	(0.34)	0.03
Total													
Patients	2293	956	(0.42)	1027	(0.45)	310	(0.14)		2939	(0.64)	1647	(0.36)	
Controls	2382	1073	(0.45)	1030	(0.43)	279	(0.12)	0.01	3176	(0.67)	1588	(0.33)	0.009[a]

Genotypic *p* was calculated by Armitage's Trend Test and allelic *p* was calculated by Fisher's exact test.
[a] Odd ratio=1.12, 95% confidence interval=1.03–1.22.

mGluR7 was the first group III mGluR found to be enriched presynaptically at active zones of hippocampal pyramidal cells (Shigemoto et al., 1996). The low affinity of mGluR7 for glutamate suggests that mGluR7 might act as a "low-pass filter" that suppresses release of glutamate only when action potentials arriving at a high frequency produce massive glutamate release. The interaction with PICK1 (protein interacting with PRKCA 1) is crucial for

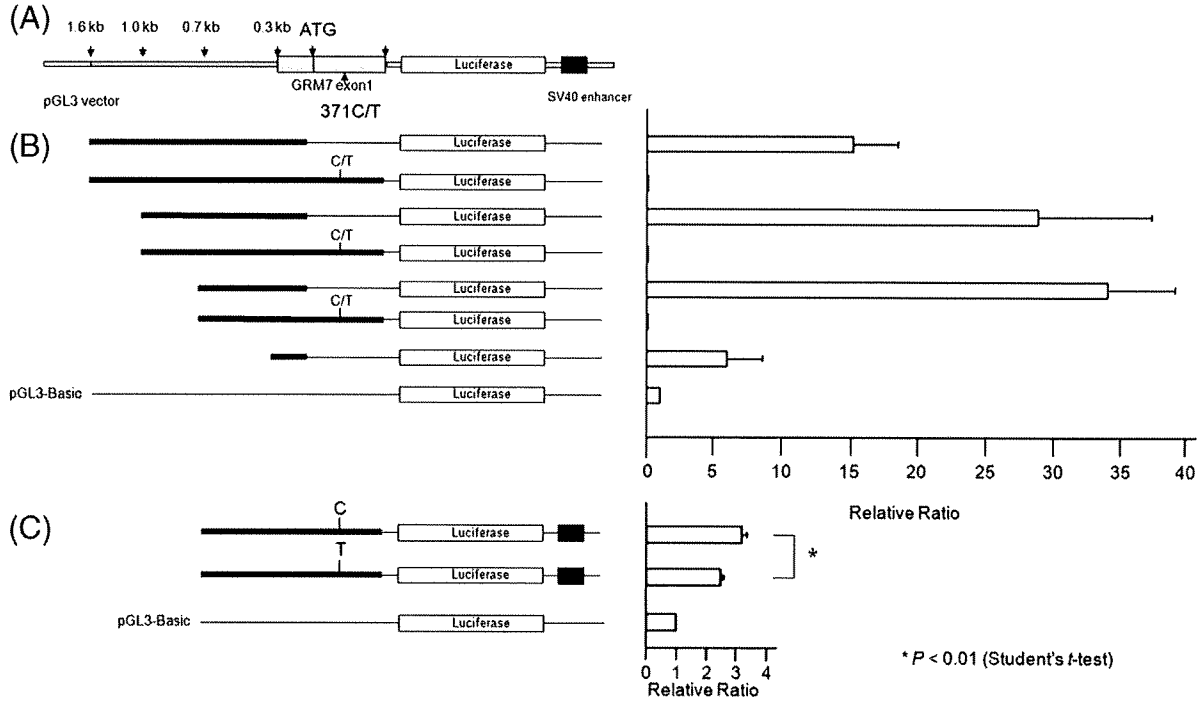

Fig. 2. Luciferase assays. (A) Schematic representation of the *GRM7* gene and reporter gene constructs. (B) Transcriptional activity of various constructs of the 5′ region and exon 1 of the human *GRM7* gene in NH-12 cells. The region indicated by fine lines were not included in the constructs. Cotransfections were performed with pRL-TK (*Renilla* luciferase) to normalize transfection efficiency. Luciferase activity was assayed 48 h after transfection. Relative ratio of expression is shown as relative to that of pGL3-Basic, which was set at 1. The mean±SEM was calculated from triplicate assays. (C) Effect of the 371C/T polymorphism on *GRM7* promoter activity. This assay was performed with pGL3-enhancer vector, because the constructs that contain *GRM7* exon 1 show extremely low luciferase activity.

the clustering of mGluR7 at presynaptic release sites (Boudin et al., 2000). *PICK1* is reported to be associated with schizophrenia (Dev and Henley, 2006; Fujii et al., 2006).

In the present study, we found an association between a functional SNP, 371T/C, in the gene encoding mGluR7 and schizophrenia. Recently, a genome-wide association study of 2000 individuals with bipolar disorder and 3000 control subjects found a strong association of SNP marker rs1485171 ($p=9.7\times10^{-5}$) in *GRM7* with bipolar disorder (Consortium, 2007). Therefore, genetic variations in the *GRM7* gene may be involved in both schizophrenia and bipolar disorder.

An association between Tyr433Phe polymorphism of *GRM7* and schizophrenia was reported (Bolonna et al., 2001). However, we failed to detect the association (allele, $p=0.33$; genotype, $p=0.63$). Recently, a copy number variation of the *GRM7* locus has been reported in patients with schizophrenia (Wilson et al., 2006). In the present study, we did not observe significant deviation from Hardy–Weinberg expectancy of the genotypic distributions of SNPs, indicating that copy number variations at the SNP examined in the present study are not common and are unlikely.

In the present study, the association of 371T/C with schizophrenia was observed in two independent case-control populations. However, its weak association (OR=1.12) requires replication studies in large sample populations of more than 2000 cases and 2000 control subjects with a power greater than 0.8. We believe that *GRM7* is an interesting target worth such studies for schizophrenia and other psychiatric disorders.

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Contributors

Author Ohtsuki ran the experiment and wrote the manuscript. Author Horiuchi and Koga prepared the sample analyzed. Author Arai, Niizato, Itokawa, Inada, Iwata, Iritani, Ozaki, Kunugi, Ujike, Watanabe, Someya, managed the sample collection. Author Ishiguro and Arinami designed this study and undertook the statistical analysis and supervised this study.

Conflict of interest

No author has conflict of interest.

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Association of polymorphisms in the haplotype block spanning the alternatively spliced exons of the *NTNG1* gene at 1p13.3 with schizophrenia in Japanese populations

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Abstract

Chromosome 1p13 is linked with schizophrenia in Japanese families, and one of the candidate genes in this region is the *netrin G1* (*NTNG1*) gene at 1p13.3. Associations of 56 tag single-nucleotide polymorphisms (SNPs) with schizophrenia were explored by transmission disequilibrium analysis in 160 Japanese trios and by case–control analysis in 2174 Japanese cases and 2054 Japanese controls. An association between SNP rs628117 and schizophrenia was identified by case–control comparison (nominal allelic $p = 0.0009$; corrected $p = 0.006$). The associated polymorphism is located in intron 9 and in the haplotype block encompassing the alternatively spliced exons of the gene. Allelic association of a different SNP in the same haplotype block in Japanese families was previously reported. These findings support that the *NTNG1* gene is associated with schizophrenia in the Japanese.

Keywords: Netrin; Schizophrenia; Alternative splicing; Association

Schizophrenia is a common disorder with a lifetime morbidity risk of 1%. A large number of family, twin, and adoption studies have revealed that individual differences in susceptibility are predominantly genetic, with a heritability of 0.70–0.85 and a 10-fold increased risk in siblings of probands [5]. Genome-wide linkage analysis of Japanese Schizophrenia Sib-Pair Linkage Group (JSSLG) samples comprising 236 Japanese families with 268 non-independent affected sib pairs with schizophrenia revealed significant evidence for linkage of schizophrenia to chromosome 1p21.2–1p13.2 (LoD = 3.39) [3]. One of the candidate genes in the chromosome 1p linkage region is *netrin G1* (*NTNG1*), which is located at 1p13.3. A potential association between a polymorphism and haplotypes with schizophrenia was reported in Japanese families [1]. This study also reported differential expression of NTNG1 isoforms between in Brodmann area 46 of postmortem brains between patients with schizophrenia and controls. The *NTNG1* gene encodes netrin G1, which is involved in the formation and/or maintenance of glutamatergic neuronal circuitry [1,6]. A case of Rett syndrome and a *de novo* translocation causing disruption of the *NTNG1* gene has been reported [2]. In the present study, we examined *NTNG1* as a candidate gene in the 1p linkage region that we previously reported.

Subjects were of Japanese descent and were recruited from the main island of Japan. Transmission disequilibrium test

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(TDT) was performed for 160 trios consisting of probands and their parents. The probands were 86 men and 74 women and the mean age ± S.D. were 29.3 ± 9.7 years. Among them, 26 trios had been included in the JSSLG linkage study. Independent case–control subjects were a total of 2174 unrelated patients with schizophrenia (mean age ± S.D.: 48.8 ± 14.5 years, 1187 men and 987 women) and 2056 mentally healthy unrelated subjects (mean age ± S.D.: 49.1 ± 14.3 years, 1109 men and 947 women) without self-reported family histories of mental illness within second-degree relatives. Consensual diagnosis was made according to DSM-IV criteria by at least two experienced psychiatrists on the basis of direct interviews, available medical records, and information provided by hospital staff and relatives. None of the patients had additional axis I disorders as defined by DSM-IV. The present study was approved by the Ethics Committees of the University of Tsukuba, Niigata University, Fujita Health University, Nagoya University, Okayama University, and Teikyo University, and all participants provided written informed consent.

Single-nucleotide polymorphisms (SNPs) were typed with the TaqMan system (Applied Biosystems, Foster City, California). Polymerase chain reaction was performed in an ABI 9700 thermocycler, and fluorescence was determined with an ABI 7900 sequence detector with single-point measurement and SDS v2.2 software (Applied Biosystems). A total of 54 tag SNPs in the *NTNG1* gene region were selected according to the Japanese genotype data of the HapMap database (http://www.hapmap.org/) and genotyped in 160 TDT trios. These tag SNPs were selected to distinguish haplotypes with minimal haplotype frequency >0.1 represented for each block. These 160 trios had approximately 80% power to detect SNP association with the use of a 5% significance given the genetic model of risk allele frequency >0.2, additive model with genotype relative risk >1.9, and r^2 capture level of the selected tag SNPs >0.8. SNPs that showed nominally significant association with schizophrenia in the TDT trios were genotyped, along with two additional SNPs, in the case–control subjects.

The two additional SNPs were genotyped to construct the haplotypes previously reported by Aoki-Suzuki et al. [1] to be associated with schizophrenia. Hardy–Weinberg equilibrium, linkage disequilibrium, and allele/haplotype frequencies, as well as association between a SNP or haplotype and schizophrenia, were analyzed with the Haploview software program (http://www.broad.mit.edu/mpg/haploview/). Permutation tests were also performed to calculate corrected p values for multiple testing by the Haploview software. Genotype-based associations were tested with the Cochran-Armitage test for trend. Statistical significance was accepted at $p < 0.05$.

SNPs screened for association with schizophrenia are listed in Table 1. Six SNPs showed nominal allelic p values <0.05, however, no SNP was significantly associated with schizophrenia after permutation tests. Among these six SNPs, rs4307594, rs7522583, and rs4364907 were found in linkage disequilibrium in each other in the TDT sample ($r^2 > 0.9$). We then examined eight SNPs in case–control subjects (Table 2). Among these, four (rs1335059, rs4364907, rs4132604, and rs628117)

Table 1
Tag SNPs in the *NTNG1* gene and p values for association with schizophrenia by TDT

SNP no.	SNP ID	Location of chromosome 1 (Mb)[a]	p	SNP no.	SNP ID	Location of chromosome 1 (Mb)[a]	p
1	rs1762482	107,311,044	0.20	28	rs7555385	107,601,690	0.37
2	rs10494059	107,354,647	0.19	29	rs4317836	107,609,420	0.91
3	rs11185061	107,372,412	0.36	30	rs12406016	107,617,150	1.00
4	rs1572281	107,372,642	0.21	31	rs10458535	107,619,635	0.73
5	rs2464046	107,377,491	0.54	32	rs11185100	107,630,361	0.94
6	rs1335059	107,386,874	0.04	33	rs6662274	107,636,384	0.11
7	rs4481881	107,398,460	0.25	34	rs6681988	107,642,407	0.12
8	rs4307594	107,405,473	0.01	35	rs10494069	107,646,688	0.40
9	rs7522583	107,412,486	0.03	36	rs2587895	107,648,435	0.93
10	rs4364907	107,417,124	0.02	37	rs920835	107,658,844	0.58
11	rs9628993	107,421,762	0.44	38	rs2218404	107,664,647	0.12
12	rs4287207	107,461,656	1.00	39	rs894904	107,667,580	0.29
13	rs5012609	107,487,420	0.95	40	rs11185112	107,676,316	1.00
14	rs10494066	107,501,550	0.20	41	rs1444040	107,676,521	0.64
15	rs3123376	107,537,908	0.64	42	rs991579	107,684,837	0.24
16	rs4132604	107,554,881	0.003	43	rs483206	107,691,721	0.36
17	rs4244127	107,561,082	0.10	44	rs1444038	107,693,358	0.77
18	rs11185086	107,563,694	1.00	45	rs628117	107,709,148	0.03
19	rs6692538	107,568,286	0.50	46	rs521721	107,713,490	0.61
20	rs12730063	107,572,878	0.27	47	rs977799	107,717,895	0.86
21	rs7411507	107,578,020	0.45	48	rs10881468	107,722,681	0.51
22	rs7531522	107,583,324	0.92	49	rs96501	107,730,889	0.36
23	rs4291528	107,586,502	0.55	50	rs597332	107,736,910	0.29
24	rs4915037	107,586,753	0.77	51	rs570718	107,742,416	0.30
25	rs6670042	107,586,818	0.94	52	rs7548775	107,745,191	0.98
26	rs6672549	107,587,001	0.16	53	rs12025693	107,760,779	0.63
27	rs7413641	107,589,827	0.43	54	rs521420	107,786,711	0.80

[a] NCBI Build 36.1.

Table 2
Case–control analysis of selected SNPs in the *NTNG1* gene

SNP no.	SNP ID	A/B allele	Population	*n*	Genotype count (frequency)			p^a	Allele count (frequency)		Allelic nominal *p*	Allelic permutation *p*
					AA	AB	BB		A	B		
6	rs1335059	A/C	Controls	566	56 (0.10)	229 (0.40)	281 (0.50)		341 (0.30)	791 (0.70)		
			Schizophrenia	571	51 (0.09)	248 (0.43)	272 (0.48)	0.79	350 (0.31)	792 (0.69)	0.82	
10	rs4364907	C/T	Controls	573	49 (0.09)	231 (0.40)	293 (0.51)		329 (0.29)	817 (0.71)		
			Schizophrenia	574	57 (0.10)	243 (0.42)	274 (0.48)	0.21	357 (0.31)	791 (0.69)	0.22	
16	rs4132604	G/T	Controls	575	166 (0.29)	301 (0.52)	108 (0.19)		633 (0.55)	517 (0.45)		
			Schizophrenia	569	192 (0.34)	266 (0.47)	111 (0.20)	0.32	650 (0.57)	488 (0.43)	0.33	
38	rs2218404	G/T	Controls	1914	1151 (0.60)	669 (0.35)	94 (0.05)		2971 (0.78)	857 (0.22)		
	G1-14		Schizophrenia	1916	1210 (0.63)	617 (0.32)	89 (0.05)	0.08	3037 (0.79)	795 (0.21)	0.08	
	rs1373336	C/T	Controls	1910	637 (0.33)	926 (0.48)	347 (0.18)		2200 (0.58)	1620 (0.42)		
	G1-17		Schizophrenia	1904	642 (0.34)	936 (0.49)	326 (0.17)	0.53	2220 (0.58)	1588 (0.42)	0.55	
	rs1444042	T/C	Controls	2056	1075 (0.52)	833 (0.41)	148 (0.07)		2983 (0.73)	1129 (0.27)		
	G1-18		Schizophrenia	2174	1079 (0.50)	884 (0.41)	211 (0.10)	0.009	3042 (0.70)	1310 (0.30)	0.006	0.04
45	rs628117	G/A	Controls	2054	146 (0.07)	833 (0.41)	1075 (0.52)		1125 (0.27)	2983 (0.73)		
			Schizophrenia	2174	213 (0.10)	908 (0.42)	1053 (0.48)	0.0009	1334 (0.31)	3014 (0.69)	0.0009	0.006
49	rs96501	C/T	Controls	1913	83 (0.04)	632 (0.33)	1198 (0.63)		798 (0.21)	3028 (0.79)		
	G1-19		Schizophrenia	1911	84 (0.04)	603 (0.32)	1224 (0.64)	0.46	771 (0.20)	3051 (0.80)	0.46	

[a] Cochran-Armitage test.

showed nominally significant association in TDT screening, and one (rs96501) was previously reported to be associated with schizophrenia [1]. Three (rs2218404, 1373336, and rs1444042) were used for constructing haplotypes previously reported to be associated with schizophrenia [1]. SNPs rs1335059, rs4364907, and rs4132604 were examined in a part of the case–control subjects consisted of 294 men and 282 women (mean age ± S.D.: 49.6 ± 14.8 years) in the patient group and 289 men and 287 women (mean age ± S.D.: 48.9 ± 12.5 years) in the control group. Among the eight SNPs, rs1444042 (nominal allelic $p = 0.006$; permutated $p = 0.04$) and rs628117 (nominal allelic $p = 0.0009$; permutated $p = 0.006$) showed significant association even after the permutation procedure. These two SNPs were in almost complete linkage disequilibrium with each other ($r^2 = 0.96$). These permutated p values were almost the same as those after Bonferroni correction for seven tests. The odds ratio for homozygous AA carriers of rs628117 against the other genotypes carrier was 1.17 (95% confidence interval, 1.04–1.32) and the odds ratio for the A allele against the G allele was 1.17 (95% confidence interval, 1.07–1.29). No apparent gender difference was observed for the association: the odds ratio for the Aallele against the G allele was 1.14 ($p = 0.06$) in the male subjects and 1.20 ($p = 0.02$) in the female subjects. Aoki-Suzuki et al. [1] reported that the minor allele frequency of rs144042 in 186 Japanese unrelated subjects (0.32) was close to that in the schizophrenia group in the present study (0.31). Although information of rs144042 is not uploaded in the JSNP database (http://snp.ims.u-tokyo.ac.jp/map/cgi-bin/searchTypingData.cgi), the minor allele frequency of rs628117, which is in linkage disequilibrium with rs144042, in 933 Japanese unrelated volunteers in the JSNP database is 0.27, similar with that in the control group in the present study.

Because rs2218404 (G1-14), rs1373336 (G1-17), and rs1444042 (G1-18) were in linkage disequilibrium (Fig. 1), and haplotype association was reported previously, we constructed haplotypes with these three SNPs. These three SNPs distinguished the common haplotypes with a frequency >0.1 in this haplotype block, according to the Japanese genotype data of the HapMap database. The global p value for the haplotype association was 0.24, and no single-haplotype association was found (Table 3).

Aoki-Suzuki et al. [1] reported association of specific haplotypes encompassing alternatively spliced exons of *NTNG1* with schizophrenia and that mRNA isoform expression differed significantly between schizophrenic and control brains [1]. In the present study, we found significant associations of two SNPs in linkage disequilibrium with schizophrenia. These SNPs are located in the haplotype block encompassing the alternatively spliced exons reported by Aoki-Suzuki et al. [1]. However, the SNPs associated with schizophrenia identified in the present study differed from that of Aoki-Suzuki et al. [1]. They reported nominal association of SNP G1-19 and association of haplotypes constructed of SNPs G1-14, G1-17, and G1-18 with schizophrenia. The associated haplotype was the fourth most common. In the present study, allelic association was observed for SNP G1-18 but not SNP G1-19. SNP G1-18 and G1-19 were not in linkage disequilibrium with each other ($D' = 0.38$, $r^2 = 0.002$). The hap-

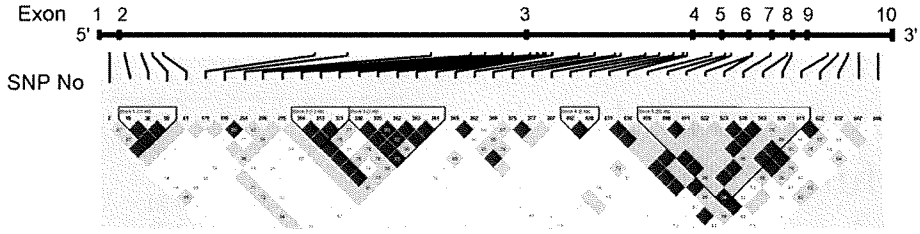

Fig. 1. Genomic structure of the *NTNG1* gene spanning 341,731 kb on chromosome 1p13.3 and linkage disequilibrium between SNPs genotyped. The SNP numbers are shown in Table 1. Linkage disequilibrium as expressed by D prime was determined with the use of Haploview software version 3.32 and haplotype blocks were determined by Gabriel's block definition.

Table 3
Haplotype association by case–control analysis

Haplotype[a]	Case frequency	Control frequency	Chi square	*p* value	HapMap JPT frequency[b]
G-T-T	0.4	0.41	0.1	0.32	0.42
G-C-C	0.28	0.26	3.83	0.05	0.25
T-C-T	0.2	0.22	3.36	0.07	0.21
G-C-T	0.09	0.09	0.31	0.58	0.07
G-T-C	0.014	0.009	2.62	0.11	0.03

[a] *NTNG1* (G1-14)-(G1-17)-(G1-18) (global $p = 0.24$).
[b] Haplotype frequency of a Japanese population in the HapMap database.

lotype association was not confirmed in the present study; the most significant associated haplotype was the second most common one (nominal $p = 0.05$), not the fourth most common one. Further studies are necessary to reconcile these differences.

We did not search for nonsynonymous variations because Aoki-Suzuki et al. [1] did not any. They evaluated isoform-specific expression of the *NTNG1* gene and found decreased expression of exon 8- and exon 9-skipping isoforms in Brodmann area 46 of postmortem brains in schizophrenia compared to that in controls. The SNPs associated with schizophrenia in the present study and the SNPs and haplotype associations reported by Aoki-Suzuki et al. [1] are located in the same haplotype block spanning alternatively spliced exons.

There were some inconsistencies between our own two samples of trio and case–control samples. The discrepancy is probably due to relatively low detection power of the 160 trio sample in which *NTNG1* was most comprehensively screened for association with schizophrenia. The four tag SNPs that reached <0.05 probability in the trios are roughly the number that would be expected by chance and these associations were not replicated in the case–control sample. Therefore, *NTNG1* is unlikely to contain common polymorphisms that exert a strong effect on schizophrenia susceptibility in Japanese samples.

Many issues remain to be addressed. Discordant findings must be reconciled by further studies with large samples. In addition, the functional significance of each mRNA isoform should be assessed, and the relation between the genetic association with schizophrenia and mRNA or protein regulation should be elucidated. Eastwood and Harrison [4] reported that expression of the *NTNG1* c isoform was reduced in schizophrenia and bipolar disorder but that the SNP rs1373336 did not affect *NTNG1* mRNA expression. The SNP rs1373336 was not found to be associated with schizophrenia in the present study.

In conclusion, results of the present study support genetic associations of polymorphisms in the haplotype block spanning the alternatively spliced exons of the *NTNG1* gene with schizophrenia.

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Letter to the Editor

Large-scale case-control study of a functional polymorphism in the *glutamate receptor, metabotropic 3* gene in patients with schizophrenia

THE A ALLELE of rs6465084, a single nucleotide polymorphism (SNP) of the *glutamate receptor, metabotropic 3* (*GRM3*) gene, has been found to be associated with decreased verbal fluency and reduced prefrontal cortical levels of N-acetylaspartate/creatine.[1] This functional SNP has been shown to be associated with schizophrenia[1] but other studies failed to demonstrate such an association.[2–5] To further investigate the inconsistent results, we conducted a case–control association study.

The Ethics Committee on Genetics of each participating institute approved the present study. All participants provided written informed consent. All participants were unrelated Japanese subjects. The subjects consisted of 2358 patients with schizophrenia, meeting the DSM-IV criteria (1273 men, 1085 women; mean age 46.8 ± 14.7 years), and 2433 control subjects (1313 men, 1120 women; mean age 45.6 ± 13.8 years). We genotyped rs6465084 (C_11245618_10; Applied Biosystems, CA, USA) using the TaqMan 5′-exonuclease assay.[6] We did not examine rs1468412,[7] rs2299225[8] and rs274622[9] because these SNP have not been shown to be associated with schizophrenia in a large Japanese sample,[5] which overlapped the subjects in the present study.

The genotype distributions did not deviate significantly from Hardy–Weinberg equilibrium in either group. The genotype and allele frequencies in patients did not differ from those in controls (Table 1). The results of the present study indicate that the rs6465084 functional polymorphism in *GRM3* does not contribute to genetic susceptibility to schizophrenia.

Table 1 Genotype and allele frequencies of rs6465084 in *GRM3*

	Patients (%)	Controls (%)	*P*
Genotype			0.39
A/A	2045 (86.7)	2132 (87.6)	
A/G	305 (12.9)	289 (11.9)	
G/G	8 (0.4)	12 (0.5)	
Allele			0.46
A	4395 (93.2)	4553 (93.6)	
G	321 (6.8)	313 (6.4)	

GRM3, glutamate receptor, metabotropic 3.

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ORIGINAL ARTICLE

Aberrant DNA methylation associated with bipolar disorder identified from discordant monozygotic twins

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To search DNA methylation difference between monozygotic twins discordant for bipolar disorder, we applied a comprehensive genome scan method, methylation-sensitive representational difference analysis (MS-RDA) to lymphoblastoid cells derived from the twins. MS-RDA isolated 10 DNA fragments derived from 5′ region of known genes/ESTs. Among these 10 regions, four regions showed DNA methylation differences between bipolar twin and control co-twin confirmed by bisulfite sequencing. We performed a case–control study of DNA methylation status of these four regions by pyrosequencing. Two regions, upstream regions of spermine synthase (*SMS*) and peptidylprolyl isomerase E-like (*PPIEL*) (CN265253), showed aberrant DNA methylation status in bipolar disorder. *SMS*, a gene on X chromosome, showed significantly higher DNA methylation level in female patients with bipolar disorder compared with control females. However, there was no difference of mRNA expression. In *PPIEL*, DNA methylation level was significantly lower in patients with bipolar II disorder than in controls. The expression level of *PPIEL* was significantly higher in bipolar II disorder than in controls. We found strong inverse correlation between gene expression and DNA methylation levels of *PPIEL*. These results suggest that altered DNA methylation statuses of *PPIEL* might have some significance in pathophysiology of bipolar disorder.
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Keywords: bipolar disorder; DNA methylation; epigenetics; gene expression; CpG island; lymphoblastoid

Introduction

Bipolar disorder is a mental disorder characterized by recurrent manic and depressive episodes affecting about 1% of the population. In spite of extensive studies, cause of bipolar disorder is unknown yet. Twin, adoption, family and linkage studies suggested that bipolar disorder is a complex disease caused by multiple genetic, environmental, or epigenetic risk factors.[1]

Epigenetics is defined as (the study of) mitotically or meiotically heritable variations in gene function that cannot be explained by changes in DNA sequence.[2] Among epigenetic mechanisms, methylation of the cytosine residue of the DNA molecule has been well studied. Methylation of CpG islands controls gene expression, genomic imprinting and X-chromosome inactivation. Abnormality of DNA methylation status is involved in various pathological process.[3–5]

Several lines of evidence suggested that epigenetics is relevant to bipolar disorder. Complex non-Mendelian inheritance, especially parent-of-origin effect, of bipolar disorder suggests the involvement of altered status of genomic imprinting and skewed X-chromosome inactivation.[6] Pharmacological studies also showed the possible role of DNA methylation in pathophysiology of bipolar disorder. Valproate, one of mood stabilizers, is known to be a histone deacetylase inhibitor.[7] Histone acethylation is coupled with DNA methylation and plays a role in the epigenetic regulation of gene expression. On the other hand, *S*-adenosyl methionine (SAM) is known to be effective for bipolar depression.[8] SAM supplies methyl residue in DNA methylation reaction, and enhance DNA methylation *in vitro*.[9] These data may suggest that epigenetic gene regulations may be relevant to the pathophysiology of bipolar disorder. Considering that DNA methylation may be involved in bipolar disorder, genome-wide screening for genes that is aberrantly methylated or demethylated in bipolar disorder is important.

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In twin studies of bipolar disorder, the concordance rate in monozygotic twins (>65%) is much higher than in dizygotic twins (>14%). However, some monozygotic twins are discordant with respect to bipolar disorder. Such discordance between monozygotic twins could be arisen from epigenetic difference.[10] In this study, we focused on a pair of monozygotic twins discordant for bipolar disorder, which was reported in the previous report.[11] We previously searched for gene expression differences between these monozygotic twins. We found that *XBP1* and its downstream gene, *HSPA5*, are commonly downregulated in the affected twins.[12] However, we could not identify differences of DNA sequence or DNA methylation of *XBP1* between the twins. Expression differences of *XBP1* might be caused by differential DNA methylation status and expression level of other genes, such as upstream genes of *XBP1*.

In this study, to comprehensively scan DNA methylation difference between the genomes of twins, we applied MS-RDA.[13] MS-RDA was developed by Ushijima *et al.*[13] and has been successfully used to identify the differences of DNA methylation status. We applied the MS-RDA method to lymphoblastoid cells derived from monozygotic twins discordant for bipolar disorder.

Materials and methods

Subjects

For MS-RDA, we examined the pair of monozygotic twins 49-year-old males discordant with respect to bipolar disorder. They were reported in a previous study.[12] The zygosity was determined by traditional phenotype markers such as blood types and HLA, micro-satellite markers and the extensive single nucleotide polymorphism (SNP) genotyping by DNA microarray (Human Mapping 50K Xba Array, Affymetrix, Santa Clara, CA, USA). For real-time quantitative RT-PCR and pyrosequencing, we examined 16 unrelated patients with bipolar I disorder (BPI) (10 men and six women, 51.2±12.6 (mean±s.d.) years old), seven unrelated patients with bipolar II disorder (BPII) (one men and six women, 62.7±9.2 years old) and 18 unrelated control subjects (12 men and six women, 45.6±12.0 years old). They were the subjects from our previous study.[14] The patients were treated with various psychotropic drugs. Three of 16 BPI patients and none of seven BPII patients were treated with valproate.

For the additional analysis, an independent set of lymphoblastoid cells derived from 14 patients with bipolar II disorder in the Fujita Health University Hospital (four women and 10 men, 39.0±13.5 (average±s.d.) years old) was used.

Consensus diagnosis was made by two psychiatrists according to the Diagnostic and Statistical Manual of Mental Disorders, Fourth Edition (DSM-IV) (American Psychiatric Association, 1994) criteria using the non-structured interview and scrutinizing medical records. Control subjects were assessed by a psychiatrist after non-structured interview, and they had no major physical or mental disorders affecting social functioning. Subjects with a history of major mental disorders, and a family history of major mental disorders within first-degree relatives were excluded from the study. All the subjects were Japanese.

Written informed consent was obtained from all the subjects. This study was approved by the ethics committees of RIKEN and participating institutions.

Lymphoblastoid cells culture

Lymphocytes from peripheral blood were transformed by Epstein–Barr virus (EBV) using standard techniques as described before.[15] Briefly, lymphocytes were separated from peripheral blood and cultured with RPMI 1640 Medium (Sigma-Aldrich, St Louis, MO, USA) containing 20% fetal bovine serum (FBS) (GIBCO, Carlsbad, Ca, USA), Penicillin and streptomycin (50 μg/ml each) (GIBCO) and supernatant of B95-8 cell culture inducted by Epstein–Barr virus. These cells were passaged every week until the cell lines were established. The cells were passaged three times a week using similar medium except for the addition of 10% FBS. The cells were kept frozen until the experiment. The blood samples of the twins were obtained on the same day, and these samples were similarly handled. The lymphoblastoid cells for the case–control analysis were used after the same number of reculture.

DNA extraction

For MS-RDA, we used genomic DNA extracted from lymphoblastoid cells of monozygotic twins discordant with respect to bipolar disorder. For pyrosequencing, we used genomic DNA extracted from lymphoblastoid cell samples that RNA was used for the real-time quantitative RT-PCR. DNA was prepared by extraction with phenol and chloroform followed by ethanol precipitation. For the analysis of peripheral blood leukocytes, the red blood cells were lysed with hypotonic buffer and genomic DNA was extracted from the pellet.

Outline of MS-RDA

MS-RDA was performed as described previously.[13] In MS-RDA, two genomes, tester and driver, were digested with *Hpa*II, a methylation sensitive restriction enzyme. *Hpa*II recognizes and digests 5′-CCGG-3′, but, is blocked by methylation of the internal C residue. After digestion, an adapter is ligated, and the entire restriction product is amplified by PCR. The PCR product is enriched with fragments originated from hypomethylated genomic regions that can be digested by HpaII. Thus, the amount of PCR product, in tester and driver, represents the DNA methylation status in each genome. After the first adapter is removed, a new adapter is ligated, only for the tester. The tester is mixed with the excess amount of driver product without the adapter. When there is no counterpart of a tester fragment in the driver, PCR

product can be amplified from the tester. If there is counterpart in the driver, PCR amplification is suppressed by the driver products, because they can hybridize with the tester fragment, but cannot be amplified by PCR because of lack of adapter sequence. By repeating this procedure twice, DNA fragments derived from hypomethylated region only in the tester genome were selectively amplified.[16]

MS-RDA

Driver and tester genomes of 10 μg each were digested with 100 units of *Hpa*II overnight. After phenol and chloroform extraction followed by ethanol precipitation, 1 μg of digestion product was ligated to 500 pmol of RHpa adapter by 800 units of T4 DNA Ligase (New England Biolabs (NEB), Berkley, MA, USA). RHpa adapter was prepared by annealing two oligonucleotides, RHpa24 and RHpa11. The ligation product was amplified by 25 cycles of PCR with RHpa24 oligonucleotide as primer as reported by Lisitsyn *et al.*[16]

The RHpa adapter of the tester and driver amplicons was removed by digestion with *Hpa*II and separation with gel filtration chromatography (CHROMA SPIN + TE-200 Columns; Clontech Laboratories Inc., Mountain View, CA, USA). The JHpa adapter (500 pmol), which was prepared by annealing JHpa24 and JHpa11, was ligated to 200 ng of the tester amplicon with T4 DNA ligase. A 200 ng of the tester DNA with the JHpa adapter at its ends was mixed with 20 μg of the driver DNA. The DNA mixture was purified by phenol and chloroform extraction and ethanol precipitation and dissolved in 4 μl of 3 × EE buffer (3 mM EDTA/3 mM *N*-(2-hydroxyethyl)pipecazine-*N*′-(3-propanesulfonic acid), pH 8.0), denatured at 96°C for 10 min and reannealed at 67°C overnight in the presence of 1 M NaCl. One-tenth of the reannealed product was amplified by PCR with the JHpa24 oligonucleotide as a primer for 10 cycles. DNA fragments linearly amplified, existing as single-stranded DNA, were digested with 100 units of Mung-Bean Nuclease, and the remaining double-stranded DNA was again amplified by PCR for 20 cycles with JHpa24 oligonucleotide.

The second cycle of competitive hybridization was performed by switching JHpa adapter to an NHpa adapter, which was prepared by annealing NHpa24 and NHpa11. A total of 20 ng of the product of the first cycle was mixed with 20 μg of driver DNA. Denaturing, reannealing and selective amplification of the self-annealed product were performed as for the first cycle. PCR products were purified by MinElute PCR Purification Kit (Qiagen, Valencia, CA, USA) and TA cloned using TOPO TA Cloning Kit (Invitrogen, Carlsbad, CA, USA). Single bacterial colonies were subject to sequencing analysis. In total, 92 independent clones were analyzed. Using the obtained sequences, a homology search was performed with the BLAST program at a GenBank Web site.

Primers were as follows: RHpa24, 5′-AGCACTCTC CAGCCTCTCACCGAC-3′; RHpa11, 5′-CGGTCGGTGAG -3′; JHpa24, 5′-ACCGACGTCGACTATCCATGAAA-C-3′; JHpa11, 5′-CGGTTTCATGG-3′; NHpa24, 5′-AGGCAA CTGTGCTATCCGAG-GGAC-3′; NHpa11, 5′-CGGTCC CTCGG-3′.

Bisulfite sequencing

After denaturation, 1 μg of genomic DNA was treated with 3.6 M sodium bisulfite. The reaction was performed at 55°C overnight. Genomic DNA was then purified with a wizard DNA clean-up system (Promega, Madison, WI, USA) and eluted with 50 μl of water. We typically used 1 μl of bisulfite-modified DNA for PCR. The CpG islands[17] were obtained through the UCSC (University of California, SantaCruz, CA, USA) Genome Browser (http://www.genome.ucsc.edu/index). Primer pairs were determined using Meth Primer software.[18] Primer pairs were as follows: PPIEL-1, 5′-TAAATTTATTTTTGGATTTAGAGTA-3′ and 5′-ACAAACTCCACAACTCTAAT-CCATT-3′; PPIEL-2, 5′-TTTAGTTTAATTTTGGTATTGTTTG-3′ and 5′-ATCTA-AAAAAAATATCCTTATTTCC-3′; phosphatidylinositol-4-phosphate 5-kinase-like 1 *(PIP5KL1)*, 5′-GGG GGTTTAAATTTGTTTAGGTTAT-3′ and 5′-CCCTTCC AAAAT-ACACAATCTAC; spermine synthase *(SMS)*, 5′-AGTGATGGAGGAGTTTTGTTAG-ATA-3′ and 5′-C CCCAAACCAAAACCCCTCTTATTT-3′; Armadillo repeat containing 3 (*ARMC3)*, 5′-AGGGTTATGAGAAGT TTTGTGGAAA-3′ and 5′-AATCAAAAAACAATTCA ACCTCAAT-3′. PCR products were purified by MinElute PCR Purification Kit, and TA cloned using TOPO TA cloning kit. Single-bacterial colonies were subject to sequencing analysis.

RNA extraction and quantitative RT-PCR

Total RNA was isolated by Trizol reagent (Invitrogen). After the DNase I treatment, 5 μg of total RNA was used for cDNA synthesis by oligo(dT) 12–18 primer and SuperScript II Reverse Transcriptase (RT) (Invitrogen).

Real-time quantitative RT-PCR using SYBR/GREEN I dye (Applied Biosystems, Foster city, CA, USA) was performed with ABI7900 (Applied Biosystems). After the denaturation at 95°C for 5 min, the PCR conditions were 95°C for 15 s and 60°C for 1 min for 50 cycles. The comparative C_t method was employed for quantification of transcripts according to the manufacture's protocol (User Bulletin #2, Applied Biosystems). Each sample was quantified in duplicate. Each experiment was repeated at least three times. Amplification of the single product was confirmed by monitoring the dissociation curve and by 3% agarose gel electrophoresis. Expression level of peptidylprolyl isomerase E-like (*PPIEL*) was also examined using commercially available cDNA (human brain parts tissue scan real-time, ORIGENE, Rockville, MD, USA). We used glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) and cofilin 1 (*CFL1*) for normalization. Primer pairs for *GAPDH* and *CFL1* have been shown previously.[19] Primer pairs used for qRT-PCR are as follows: *PPIEL*, 5′-TCGTGCCCATGACCACAG AG-3′ and 5′-CGGTGGAAGCTGCTTCCCTT-3; *SMS*, 5′-TCCAATCTCCACGTCTCCAGAA-3′ and 5′-TGTCA GATTGACACAGTT-CCCCTG-3′.