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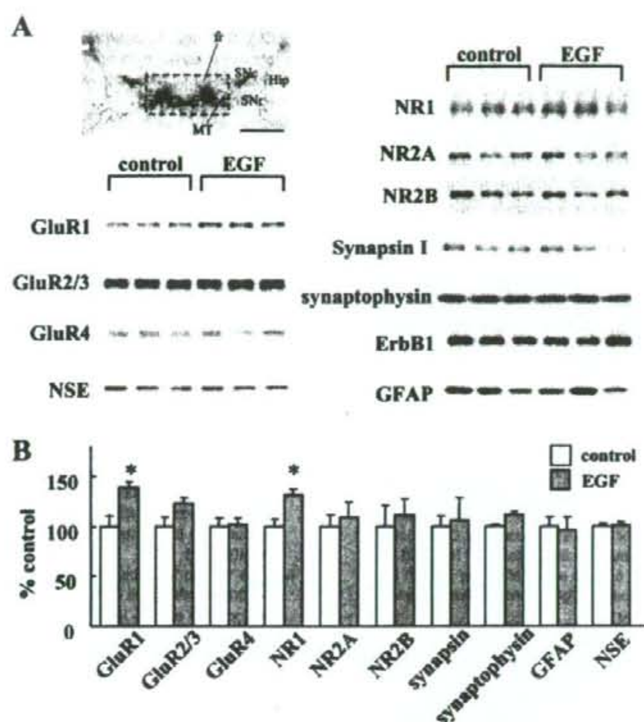

Fig. 6. Protein expression of glutamate receptor subunits in the VTA. After daily injection of saline or EGF, brain tissue around the VTA including SNc was dissected at P15 as shown in the schematic outline of the midbrain immunostained with the anti–tyrosine hydroxylase antibody (A). This inset in A includes fr, MT, hippocampus (Hip), and VTA. Scale bar=1 mm for inset in A. Immunoblots of 5 or 20 μg protein were probed with antibodies raised against AMPA receptor subunits (GluR1, GluR2/3, and GluR4) and NMDA receptor subunits (NR1, NR2A, and NR2B). Immunoreactivity for the presynaptic proteins synapsin I and synaptophysin, the neuronal marker NSE, the astrocyte marker GFAP, and ErbB1, was examined as well. Representative immunoblots are displayed. (B) Levels of immunoreactivity were measured by densitometry (n=5, each represents a pooled sample of two mice). Results are all normalized to protein levels in controls (100%) and plotted. * P<0.05, t-test.

the expression of the presynaptic proteins synapsin I and synaptophysin was not affected by EGF. Thus, EGF has no apparent influences on neurotransmitter release from afferent terminals in the VTA.

In situ hybridization reveals the expression of ErbB1 mRNA in rat midbrain (Seroogy et al., 1994; Kornblum et al., 1997). EGF circulating in peripheral blood can cross the blood–brain barrier (Pan and Kastin, 1999; Kastin et al., 1999) and activate ErbB1 in the brain (Futamura et al., 2003). In particular, the blood–brain barrier is leaky during early postnatal stage of rodents when the blood–brain barrier is not fully established (Tohmi et al., 2007). In agreement, biotinylated EGF efficiently penetrated the blood–brain barrier of neonatal mice and reached the midbrain region. Peripherally administered EGF also triggered phosphorylation of ErbB1 as well as that of ErbB2 in the ventral midbrain tissue. It is possible that ErbB1 forms hetero-oligomers with ErbB2 in this brain region (Leahy, 2004) and phosphorylates ErbB2 (Fox and Kornblum, 2005; Gerecke et al., 2001). These results illustrate that circulating EGF must have significant impact on the midbrain dopaminergic system, at least, during early postnatal development.

Rodents treated with EGF as neonates exhibit schizophrenia-like behavioral abnormalities in prepulse inhibition, exploratory motor activity, and social interaction at the adult stages (Futamura et al., 2003; Tohmi et al., 2005; Sotoyama et al., 2007). These behavioral abnormalities induced by EGF may in part result from the alteration in the dopaminergic system (Sotoyama et al., 2007). Consistent with these observations, EGF-treated rats exhibits higher sensitivity to cocaine, which enhances glutamatergic neurotransmission to dopamine neurons (Ungless et al., 2001; Zhang et al., 1997; Mizuno et al., 2004). Cocaine facilitates AMPA receptor-mediated transmission in the VTA (Zhang et al., 1997; Ungless et al., 2001), enhances agonist-induced burst firing, and dopamine release in the nucleus accumbens, leading to behavioral and cognitive impairments (Tong et al., 1995; Giorgetti et al., 2001). The present findings suggest that neonatal EGF treatment may mimic the pharmacological action of cocaine.

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The present synaptic effects of EGF on dopaminergic neurons appear not to be persistent, however. At P30, when 2 weeks passed after completion of EGF administration, there were no detectable influences remaining in the synaptic properties of dopaminergic neurons as well as in glutamate receptor expressions in the midbrain (H. Nawa, unpublished observations). In this context, further studies should determine how the change in the synaptic properties of dopaminergic neurons leads to the behavioral abnormalities at the adult stage.

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REFERENCES

Abe K, Xie FJ, Saito H (1991) Epidermal growth factor enhances short-term potentiation and facilitates induction of long-term potentiation in rat hippocampal slices. Brain Res 547:171–174.

Abe K, Saito H (1992) Epidermal growth factor selectively enhances NMDA receptor-mediated increase of intracellular Ca^{2+} concentration in rat hippocampal neurons. Brain Res 587:102–108.

Alexi T, Hefti F (1993) Trophic actions of transforming growth factor alpha on mesencephalic dopaminergic neurons developing in culture. Neuroscience 55:903–918.

Birecree E, King LE Jr, Nanney LB (1991) Epidermal growth factor and its receptor in the developing human nervous system. Dev Brain Res 60:145–154.

Blum M (1998) A null mutation in TGF-alpha leads to a reduction in midbrain dopaminergic neurons in the substantia nigra. Nat Neurosci 1:374–377.

Bonci A, Malenka RC (1999) Properties and plasticity of excitatory synapses on dopaminergic and GABAergic cells in the ventral tegmental area. J Neurosci 19:3723–3730.

Casper D, Blum M (1995) Epidermal growth factor and basic fibroblast growth factor protect dopaminergic neurons from glutamate toxicity in culture. J Neurochem 65:1016–1026.

Casper D, Mytilineou C, Blum M (1991) EGF enhances the survival of dopamine neurons in rat embryonic mesencephalon primary cell culture. J Neurosci Res 30:372–381.

Chen LW, Wei LC, Lang B, Ju G, Chan YS (2001) Differential expression of AMPA receptor subunits in dopamine neurons of the rat brain: a double immunocytochemical study. Neuroscience 106:149–160.

Ferrari G, Toffano G, Skaper SD (1991) Epidermal growth factor exerts neuronotrophic effects on dopaminergic and GABAergic CNS neurons: comparison with basic fibroblast growth factor. J Neurosci Res 30:493–497.

Ford CP, Mark GP, Williams JT (2006) Properties and opioid inhibition of mesolimbic dopamine neurons vary according to target location. J Neurosci 26:2788–2797.

Fox IJ, Kornblum HI (2005) Developmental profile of ErbB receptors in murine central nervous system: implications for functional interactions. J Neurosci Res 79:584–597.

Fukunaga K, Horikawa K, Shibata S, Takeuchi Y, Miyamoto E (2002) Ca^{2+}/calmodulin-dependent protein kinase II-dependent long-term potentiation in the rat suprachiasmatic nucleus and its inhibition by melatonin. J Neurosci Res 70:799–807.

Futamura T, Toyooka K, Iritani S, Niizato K, Nakamura R, Tsuchiya K, Someya T, Kakita A, Takahashi H, Nawa H (2002) Abnormal expression of epidermal growth factor and its receptor in the forebrain and serum of schizophrenic patients. Mol Psychiatry 7:673–682.

Futamura T, Kakita A, Tohmi M, Sotoyama H, Takahashi H, Nawa H (2003) Neonatal perturbation of neurotrophic signaling results in abnormal sensorimotor gating and social interaction in adults: implication for epidermal growth factor in cognitive development. Mol Psychiatry 8:19–29.

Geisler S, Derst C, Veh RW, Zahm DS (2007) Glutamatergic afferents of the ventral tegmental area in the rat. J Neurosci 27:5730–5743.

Gerecke KM, Wyss JM, Karavanova I, Buonanno A, Carroll SL (2001) ErbB transmembrane tyrosine kinase receptors are differentially expressed throughout the adult rat central nervous system. J Comp Neurol 433:86–100.

Giorgetti M, Hotsenpiller G, Ward P, Teppen T, Wolf ME (2001) Amphetamine-induced plasticity of AMPA receptors in the ventral tegmental area: effects on extracellular levels of dopamine and glutamate in freely moving rats. J Neurosci 21:6362–6369.

Hatanaka H, Arimatsu Y (1984) Monoclonal antibodies to tyrosine hydroxylase from rat pheochromocytoma PC12h cells with special reference to nerve growth factor-mediated increase of the immunoprecipitable enzymes. Neurosci Res 1:253–263.

Ikeda Y, Yahata N, Ito I, Nagano M, Toyota T, Yoshikawa T, Okubo Y, Suzuki H (2008) Low serum levels of brain-derived neurotrophic factor and epidermal growth factor in patients with chronic schizophrenia. Schizophr Res 101:58–66.

Ishiyama J, Saito H, Abe K (1991) Epidermal growth factor and basic fibroblast growth factor promote the generation of long-term potentiation in the dentate gyrus of anaesthetized rats. Neurosci Res 12:403–411.

Iwakura Y, Piao YS, Mizuno M, Takei N, Kakita A, Takahashi H, Nawa H (2005) Influences of dopaminergic lesion on epidermal growth factor-ErbB signals in Parkinson's disease and its model: neurotrophic implication in nigrostriatal neurons. J Neurochem 93:974–983.

Johnson SW, North RA (1992) Two types of neurone in the rat ventral tegmental area and their synaptic inputs. J Physiol 450:455–468.

Kastin AJ, Pan W, Maness LM, Banks WA (1999) Peptides crossing the blood-brain barrier: some unusual observations. Brain Res 848:96–100.

Kim SO, Houtman JC, Jiang J, Ruppert JM, Bertics PJ, Frank SJ (1999) Growth hormone-induced alteration in ErbB-2 phosphorylation status in 3T3-F442A fibroblasts. J Biol Chem 274:36015–36024.

Kornblum HI, Hussain RJ, Bronstein JM, Gall CM, Lee DC, Seroogy KB (1997) Prenatal ontogeny of the epidermal growth factor receptor and its ligand, transforming growth factor alpha, in the rat brain. J Comp Neurol 380:243–261.

Lazar LM, Blum M (1992) Regional distribution and developmental expression of epidermal growth factor and transforming growth factor-alpha mRNA in mouse brain by a quantitative nuclease protection assay. J Neurosci 12:1688–1697.

Leahy DJ (2004) Structure and function of the epidermal growth factor (EGF/ErbB) family of receptors. Adv Protein Chem 68:1–27.

Liang F, Hatanaka Y, Saito H, Yamamori T, Hashikawa T (2000) Differential expression of gamma-aminobutyric acid type B receptor-1a and -1b mRNA variants in GABA and non-GABAergic neurons of the rat brain. J Comp Neurol 416:475–495.

Margolis EB, Lock H, Hjelmstad GO, Fields HL (2006) The ventral tegmental area revisited: is there an electrophysiological marker for dopaminergic neurons? J Physiol 577:907–924.

Mizuno M, Malta RS Jr, Nagano T, Nawa H (2004) Conditioned place preference and locomotor sensitization after repeated administration of cocaine or methamphetamine in rats treated with epidermal growth factor during the neonatal period. Ann N Y Acad Sci 1025:612–618.

Mizuno M, Sotoyama H, Narita E, Kawamura H, Namba H, Zheng Y, Eda T, Nawa H (2007) A cyclocoxkinase-2 inhibitor ameliorates behavioral impairments induced by striatal administration of epidermal growth factor. J Neurosci 27:10116–10127.

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Nagano T, Namba H, Abe Y, Aoki H, Takei N, Nawa H (2007) In vivo administration of epidermal growth factor and its homologue attenuates developmental maturation of functional excitatory synapses in cortical GABAergic neurons. Eur J Neurosci 25:380–390.

Neuhoff H, Neu A, Liss B, Roeper J (2002) I(h) channels contribute to the different functional properties of identified dopaminergic subpopulations in the midbrain. J Neurosci 22:1290–1302.

Nugent FS, Hwong AR, Udaka Y, Kauer JA (2008) High-frequency afferent stimulation induces long-term potentiation of field potentials in the ventral tegmental area. Neuropsychopharmacology 33:1704–1712.

Obata K, Nishiye H, Fujita SC, Shirao T, Inoue H, Uchizono K (1986) Identification of a synaptic vesicle-specific 38,000-dalton protein by monoclonal antibodies. Brain Res 375:37–48.

Pan W, Kastin AJ (1999) Entry of EGF into brain is rapid and saturable. Peptides 20:1091–1098.

Paquet M, Tremblay M, Soghomonian JJ, Smith Y (1997) AMPA and NMDA glutamate receptor subunits in midbrain dopaminergic neurons in the squirrel monkey: an immunohistochemical and in situ hybridization study. J Neurosci 17:1377–1396.

Patterson PH, Nawa H (1993) Neuronal differentiation factors/cytokines and synaptic plasticity. Cell 72(Suppl):123–137.

Paxinos G, Franklin KBJ (2004) The mouse brain in stereotaxic coordinates, 2nd edition. San Diego: Academic Press.

Pezzoli G, Zecchinelli A, Ricciardi S, Burke RE, Fahn S, Scarlato G, Carenzi A (1991) Intraventricular infusion of epidermal growth factor restores dopaminergic pathway in hemiparkinsonian rats. Mov Disord 6:281–287.

Piao YS, Iwakura Y, Takei N, Nawa H (2005) Differential distributions of peptides in the epidermal growth factor family and phosphorylation of ErbB 1 receptor in adult rat brain. Neurosci Lett 390:21–24.

Plata-Salaman CR (1991) Epidermal growth factor and the nervous system. Peptides 12:653–663.

Schaudies RP, Christian EL, Savage CR Jr (1989) Epidermal growth factor immunoreactive material in the rat brain. Localization and identification of multiple species. J Biol Chem 264:10447–10450.

Seroogy KB, Numan S, Gall CM, Lee DC, Kornblum HI (1994) Expression of EGF receptor mRNA in rat nigrostriatal system. Neuroreport 6:105–108.

Sotoyama H, Namba H, Takei N, Nawa H (2007) Neonatal exposure to epidermal growth factor induces dopamine D2-like receptor supersensitivity in adult sensorimotor gating. Psychopharmacology (Berl) 191:783–792.

Tohmi M, Tsuda N, Mizuno M, Takei N, Frankland PW, Nawa H (2005) Distinct influences of neonatal epidermal growth factor challenge on adult neurobehavioral traits in four mouse strains. Behav Genet 35:615–629.

Tohmi M, Tsuda N, Zheng Y, Mizuno M, Sotoyama H, Shibuya M, Kawamura M, Kakita A, Takahashi H, Nawa H (2007) The cellular and behavioral consequences of interleukin-1 alpha penetration through the blood-brain barrier of neonatal rats: a critical period for efficacy. Neuroscience 150:234–250.

Tong ZY, Overton PG, Clark D (1995) Chronic administration of (+)-amphetamine alters the reactivity of midbrain dopaminergic neurons to prefrontal cortex stimulation in the rat. Brain Res 674:63–74.

Ungless MA, Whistler JL, Malenka RC, Bonci A (2001) Single cocaine exposure in vivo induces long-term potentiation in dopamine neurons. Nature 411:583–587.

Wanat MJ, Hopf FW, Stuber GD, Phillips PE, Bonci A (2008) Corticotropin-releasing factor increases mouse ventral tegmental area dopamine neuron firing through a protein kinase C-dependent enhancement of Ih. J Physiol 586:2157–2170.

Watakabe A, Ohsawa S, Hashikawa T, Yamamori T (2006) Binding and complementary expression patterns of semaphorin 3E and plexin D1 in the mature neocortices of mice and monkeys. J Comp Neurol 499:258–273.

White FJ (1996) Synaptic regulation of mesocorticolimbic dopamine neurons. Annu Rev Neurosci 19:405–436.

Young ID, Ailles L, Deugau K, Kisileusky (1991) Transcription of cRNA for in situ hybridization from polymerase chain reaction-amplified DNA. Lab Invest 64:709–712.

Yung KK (1998) Localization of ionotropic and metabotropic glutamate receptors in distinct neuronal elements of the rat substantia nigra. Neurochem Int 33:313–326.

Zhang XF, Hu XT, White FJ, Wolf ME (1997) Increased responsiveness of ventral tegmental area dopamine neurons to glutamate after repeated administration of cocaine or amphetamine is transient and selectively involves AMPA receptors. J Pharmacol Exp Ther 281:699–706.

Zheng Y, Sudou K, Nawa H, Namba H (2006) Field potential recording in the ventral tegmental area: pharmacological and toxicological evaluations of postsynaptic dopaminergic neuron activity. Neurosci Res 55:426–433.

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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 $\pm$ 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.

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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

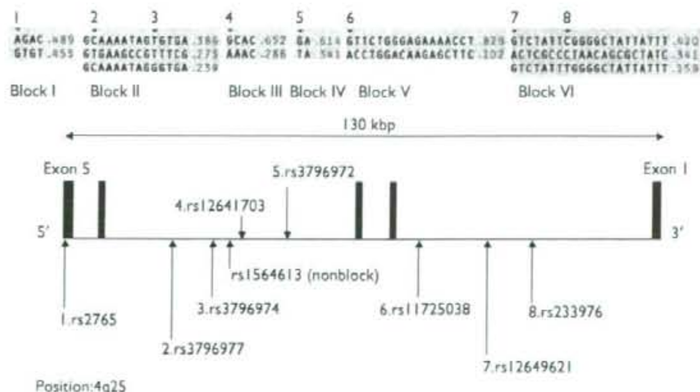

Fig. 1 Genomic structure of *TACR3* with haplotype tag single nucleotide polymorphisms (SNPs) and haplotype frequencies in each linkage disequilibrium block provided by HapMap database V.16. Numbers under or above the arrows represent the SNPs we selected in this study.

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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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References

1. Almeida TA, Rojo J, Nieto PM, Pinto FM, Hernandez M, Martin JD, *et al.* Tachykinins and tachykinin receptors: structure and activity relationships. *Curr Med Chem* 2004; **11**:2045–2081.
2. Humpel C, Saria A, Regoli D. Injection of tachykinins and selective neurokinin receptor ligands into the substantia nigra reticulata increases striatal dopamine and 5-hydroxytryptamine metabolism. *Eur J Pharmacol* 1991; **195**:107–114.
3. Keegan KD, Woodruff GN, Pinnock RD. The selective NK3 receptor agonist senktide excites a subpopulation of dopamine-sensitive neurones in the rat substantia nigra pars compacta *in vitro*. *Br J Pharmacol* 1992; **105**:3–5.
4. Stoessl AJ, Szczutkowski E, Glenn B, Watson I. Behavioural effects of selective tachykinin agonists in midbrain dopamine regions. *Brain Res* 1991; **565**:254–262.
5. Spooren W, Riemer C, Meltzer H. Opinion: NK3 receptor antagonists: the next generation of antipsychotics? *Nat Rev Drug Discov* 2005; **4**:967–975.
6. Stoessl AJ, Dourish CT, Young SC, Williams BJ, Iversen SD, Iversen LL. Senktide, a selective neurokinin B-like agonist, elicits serotonin-mediated behaviour following intracisternal administration in the mouse. *Neurosci Lett* 1987; **80**:321–326.
7. Stoessl AJ, Dourish CT, Iversen SD. The NK-3 tachykinin receptor agonist senktide elicits 5-HT-mediated behaviour following central or peripheral administration in mice and rats. *Br J Pharmacol* 1988; **94**:285–287.
8. Meltzer HY, Arvanitis L, Bauer D, Rein W. Placebo-controlled evaluation of four novel compounds for the treatment of schizophrenia and schizoaffective disorder. *Am J Psychiatry* 2004; **161**:975–984.
9. Barrett JC, Fry B, Maller J, Daly MJ. Haploview: analysis and visualization of LD and haplotype maps. *Bioinformatics* 2005; **21**: 263–265.
10. Motsinger AA, Lee SL, Mellick G, Ritchie MD. GPNN: power studies and applications of a neural network method for detecting gene–gene interactions in studies of human disease. *BMC Bioinformatics* 2006; **7**:39.
11. Sumiyoshi T, Matsui M, Yamashita I, Nohara S, Kurachi M, Uehara T, *et al.* The effect of tandospirone, a serotonin(1A) agonist, on memory function in schizophrenia. *Biol Psychiatry* 2001; **49**:861–868.

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Genetic analysis of the gene coding for DARPP-32 (*PPP1R1B*) in Japanese patients with schizophrenia or bipolar disorder

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Abstract

Several lines of evidence, including genome-wide linkage scans and postmortem brain studies of patients with schizophrenia or bipolar disorder, have suggested that DARPP-32 (dopamine- and cAMP-regulated phosphoprotein, 32 kDa), a key regulatory molecule in the dopaminergic signaling pathway, is involved in these disorders. After evaluating the linkage disequilibrium pattern of the gene encoding DARPP-32 (*PPP1R1B*; located on 17q12), we conducted association analyses of this gene with schizophrenia and bipolar disorder. Single-marker and *haplotypic* analyses of four single nucleotide polymorphisms (SNPs; rs879606, rs12601930, rs907094, and rs3764352) in a sample set (subjects with schizophrenia=384, subjects with bipolar disorder=318, control subjects=384) showed that *PPP1R1B* polymorphisms were not significantly associated with schizophrenia, whereas, even after Bonferroni corrections, significant associations with bipolar disorder were observed for rs12601930 (corrected genotypic $p=0.00059$) and rs907094 (corrected allelic $p=0.040$). We, however, could not confirm these results in a second independent sample set (subjects with bipolar disorder=366, control subjects=370). We now believe that the significant association observed with the first sample set was a result of copy number aberrations in the region surrounding these SNPs. Our findings suggest that *PPP1R1B* SNPs are unlikely to be related to the development of schizophrenia and bipolar disorder in the Japanese population.
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Keywords: Schizophrenia; Bipolar disorder; Dopamine- and cAMP-regulated phosphoprotein; 32 kDa; Japanese population

1. Introduction

A number of studies have proposed that disruption of monoaminergic pathways, and in particular the dopaminergic pathway, contributes to both schizophrenia and bipolar disorder (Catapano and Manji, 2007; Murray

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et al., 2004). DARPP-32 (dopamine- and cAMP-regulated phosphoprotein, 32 kDa), a critical molecule in *the* striatal neurons, regulates the dopaminergic signaling pathway through phosphorylation of protein phosphatase-1 and protein kinase A (Fienberg et al., 1998). Recently, it has been revealed that DARPP-32 also plays an important role in the regulation of *glutamatergic* signaling pathway (Nishi et al., 2005), which is also thought to contribute to the development of these disorders (Beneyto et al., 2007; Svenningsson et al., 2003).

DARPP-32 knockout mice have been shown to have abnormal responses to psychoactive drugs, such as the decrease of cage climbing behavior induced by dopamine agonists (Fienberg et al., 1998) and the decrease of attenuating effect of antidepressants on immobility (Svenningsson et al., 2002).

Moreover, reduced expression of DARPP-32 has been observed in the postmortem brain of schizophrenic patients (Albert et al., 2002). This is suggested to be related to neostriatal volume, activation, and functional connectivity in the prefrontal cortex, all of which are thought to be abnormal in patients with schizophrenia (Meyer-Lindenberg et al., 2007).

Additionally, several lines of evidence have demonstrated that genetic factors contribute to the development of schizophrenia and bipolar disorder, and genome-wide linkage scans have shown that several chromosomal regions are simultaneously linked to the development of these disorders. Namely, a chromosomal region within 17q, which includes the gene encoding DARPP-32 (*PPP1R1B*; located on 17q12), has been demonstrated to have high logarithm of the odds scores for schizophrenia (Cardno et al., 2001) and bipolar disorder (Dick et al., 2003), i.e. 2.54 and 3.63, respectively.

Therefore, *PPP1R1B* is considered to be one of the candidate genes that contribute to these disorders. In the present study, we performed linkage disequilibrium analysis of *PPP1R1B*, and investigated the association of polymorphisms in this gene with schizophrenia and bipolar disorder in Japanese patients. We employed a two-stage analysis using two independent sets of samples as a previous report (Ikeda et al., 2005). Additionally, copy number variations (CNVs), which have been observed for many genes (Lee and Lupski, 2006; Redon et al., 2006) can affect the accuracy of genotyping with single nucleotide polymorphisms (SNPs). Therefore, we also explored copy number differences of this gene to test the accuracy of genotyping with the SNPs, which deviated from the Hardy–Weinberg equilibrium (HWE).

2. Materials and methods

2.1. Subjects

The subjects for the case-control analysis consisted of 384 patients with schizophrenia (226 males and 158 females; 52.1 ± 15.3 years old), 318 patients with bipolar disorder (162 males and 156 females; 44.0 ± 20.7 years old), and 384 control subjects (159 males and 225 females; 43.9 ± 15.9 years old). To confirm a significant association with bipolar disorder, a second sample set was used, which consisted of 366 patients with bipolar disorder (181 males and 185 females; 50.1 ± 13.4 years old), and 370 control subjects (185 males and 185 females; 50.6 ± 12.6 years old).

For the analysis of copy number differences, we selected 12 male and 12 female subjects (schizophrenia patients: 54.9 ± 12.5 years old; bipolar disorder patients: 46.3 ± 17.4 years old; control subjects: 43.0 ± 12.6 years old) for screening, and another independent sample set of 36 male and 36 female subjects (schizophrenia patients: 41.1 ± 12.2 years old; bipolar disorder patients: 47.1 ± 15.6 years old; control subjects: 43.5 ± 12.7 years old) was used to confirm the results. The subjects used for the copy number analysis were also included in the first sample set used for the association study.

All subjects were unrelated and ethnically Japanese. The patients were diagnosed by at least two experienced psychiatrists according to the DSM-IV-TR (Diagnostic and Statistical Manual of Mental Disorders, Fourth Edition, Text Revision) criteria for schizophrenia and bipolar disorder on the basis of unstructured interviews and reviews of their medical records. All healthy control subjects were also psychiatrically screened on the basis of unstructured interviews.

This study was approved by the Ethics Committees of the Nagoya University Graduate School of Medicine, Fujita Health University, and the RIKEN Brain Science Institute. Written informed consent was obtained from each subject.

2.2. Linkage disequilibrium (LD) analysis and tagging SNP selection

For LD analysis, we consulted the HapMap database (release #21a; population: Japanese in Tokyo; minor allele frequency: more than 0.05) in order to obtain SNPs throughout the entire coding region of *PPP1R1B* (GenBank accession No. NM_032192) as well as in flanking regions 500 base pairs (bp) upstream and 500 bp downstream of the coding regions. For the gene-spanning analysis, we selected four SNPs (rs2271309,

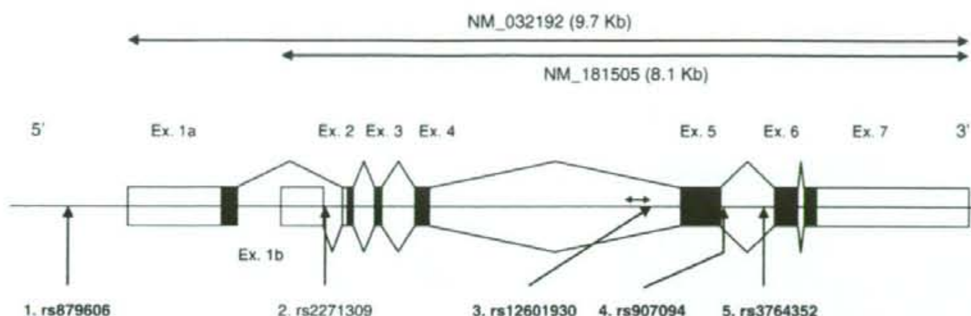

Fig. 1. Genomic structure of *PPP1R1B*. Black boxes indicate protein-coding regions, whereas open boxes denote untranslated regions (UTR). Each box represents the *PPP1R1B* exons. Numbers under the arrows represent the SNP IDs. Bold numbers represent tagging SNPs (pairwise tagger; $r^2>0.8$; Haploview 3.32). All SNPs in the coding region are listed as well as those within the 500-bp upstream 5′-flanking region and the 500-bp downstream 3′ UTR of *PPP1R1B*. Arrows ~200 bp upstream of rs12601930 shows the sites that were PCR amplified for copy number analysis.

rs12601930, rs907094, and rs3764352) in addition to a dbSNP (rs879606) located in the promoter region (Fig. 1). After evaluating the LD pattern with 48 control subjects using Haploview version 3.32, rs2271309 was excluded according to the criterion for pairwise tagging, $r^2>0.8$ (de Bakker et al., 2005) (see Fig. 2).

2.3. SNP genotyping

Genotyping of tagging SNPs was carried out primarily using polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) analysis. TaqMan assays were used when available (Applied Biosystems, California, USA). TaqMan probes and Universal PCR Master Mix were obtained from Applied Biosystems. Allelic-specific fluorescence was measured using an ABI PRISM 7900 Sequence Detector System (Applied Biosystems). Information about each primer pair and enzyme is available upon request.

2.4. Quantitative real-time PCR

The *PPP1R1B* copy number was analyzed using real-time PCRs with a specific primer set and FAM-labeled fluorescent probe or TaqMan expression assays (Applied Biosystems). The test region for *PPP1R1B* was ~200 bp upstream of the SNP that produced results that deviated from the HWE (Fig. 1) and the prostaglandin transporter gene (*SLCO2A1*) was used as a single-copy control gene (Wilson et al., 2006). We did not use glucose-6-phosphate dehydrogenase (G6PD), which is located on the X chromosome and was used as single-copy control gene in the study from Wilson et al., because it showed an unstable copy number value (not an integral value) in their report.

To determine the relative copy number, 10 ng of genomic DNA was assayed in triplicate in 20 μL of reaction solution containing 1 × final concentration TaqMan Universal Master Mix (Applied Biosystems) and 1 × final concentration TaqMan probe (Applied Biosystems) specific for *PPP1R1B* or 700 nM each primer and 200 nM probe specific for *SLCO2A1*.

Each experiment was performed using an ABI PRISM 7900 Sequence Detector System (Applied Biosystems). To evaluate the relative copy number of *PPP1R1B*, we calculated the relative quantity of the dose of *PPP1R1B* using a comparative C_T method (*PPP1R1B* vs. *SLCO2A1*). The TaqMan specific primers and FAM-labeled fluorescent probe used for the PCR amplifications were as follows: *PPP1R1B*-FAM probe (5′-FAM-CCCCTTGCTCCTTTCC-MGBNFQ-3′), *PPP1R1B*-for (5′-GCCTTGGCCCCTTTCTCTAA-3′), *PPP1R1B*-rev (5′-GCAGCTGGAGACAAGTTTCC-3′), *SLCO2A1*-FAM (5′-FAM-CCATCCATGTCCTCATCTC-

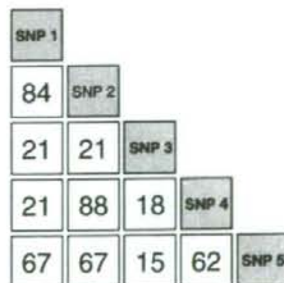

Fig. 2. LD analysis of *PPP1R1B*. Numbers in the top gray boxes correspond to the SNP ID numbers in Fig. 1. Numbers in the white boxes represent the r^2 values after the decimal point.

Table 1
Genotype and allele frequency of *PPP1R1B* in schizophrenia and controls

Gene symbol	SNP ID (M/m)	Method of genotyping	Genotype[a]						p value	Armitage's	Allele				p value	Global
			SCZ[b]			CON[b]				p value	SCZ		CON			p value[c]
PPP1R1B	rs879606 (G/A)	RFLP	G/G 62 (34%)	G/A 81 (45%)	A/A 39 (21%)	G/G 58 (32%)	G/A 91 (49%)	A/A 35 (19%)	0.631	0.985	G 205 (56%)	A 159 (44%)	G 207 (56%)	A 161 (44%)	0.985	0.697
	Rs12601930 (C/T)	real time PCR	C/C 256 (68%)	C/T 110 (29%)	T/T 11 (3%)	C/C 240 (63%)	C/T 134 (35%)	T/T 8 (2%)	0.190	0.271	C 622 (83%)	T 132 (18%)	C 614 (80%)	T 150 (20%)	0.287	
	rs907094 (T/C)	real time PCR	T/T 99 (27%)	T/C 202 (54%)	C/C 71 (19%)	T/T 114 (30%)	T/C 193 (50%)	C/C 77 (20%)	0.518	0.673	T 400 (54%)	C 344 (46%)	T 421 (55%)	C 347 (45%)	0.681	
	rs3764352 (A/G)	RFLP	A/A 86 (24%)	A/G 193 (54%)	G/G 77 (22%)	A/A 104 (28%)	A/G 195 (52%)	G/G 79 (21%)	0.582	0.419	A 365 (51%)	G 347 (49%)	A 403 (53%)	G 353 (47%)	0.434	

[a]M: major allele, m: minor allele [b]SCZ: schizophrenia, CON: control [c]global p value: haplotypic analysis.

MGBNFQ-3′), *SLCO2A1*-for (5′-ATCCCCAAAG-CACCTGGTTT-3′), and *SLCO2A1*-rev (5′-AGAGGC-CAAGATAGTCCTGGTAA-3′).

2.5. Statistical analysis

Genotype deviations from the HWE and single-marker association were analyzed using Haploview software. We evaluated the allelic and genotypic associations by the χ^2-test. Genotypic association of SNPs that deviated from the HWE was analyzed using Cochran-Armitage trend tests for multiplicative model of inheritance (Balding, 2006). Haplotypic analyses were performed with Unphased version 2.403 (Dudbridge, 2003). The significance level for all statistical tests was 0.05. Bonferroni corrections were used for multiple comparisons. Power calculations were performed using the genetic statistical package on a genetic power calculator (Purcell et al., 2003).

3. Results

The genotype and allele frequency of each SNP in schizophrenic patients, bipolar disorder patients, and control subjects are summarized in Table 1 and Table 2-1, respectively. The observed genotype frequencies of the tagging SNPs were within the distribution expected from the HWE except for rs12601930.

Neither the genotype nor the allele frequency of any of the examined *PPP1R1B* SNPs in the schizophrenic patients differed significantly from those observed for the control subjects (Table 1). Moreover, the distribution of haplotypic frequencies in the schizophrenia patients did not differ significantly from that in control subjects. Power analysis showed that more than 80% power in detecting an association with schizophrenia was obtained when the genotype relative risk (GRR) was set from 1.35 to 1.51 in a multiplicative model of inheritance.

A significant association was observed with both the genotype and the allele containing rs907094 ($p=0.036$ and $p=0.010$, respectively), whereas a significant association was only found with the genotype for rs12601930 ($p=0.000147$). Haplotypic analysis supported this association (global $p=0.030$; Table 2-1). After Bonferroni corrections, the observed positive associations were no longer significant for the rs907094 genotype (corrected $p=0.144$) and haplotype (corrected global $p=0.120$), whereas, even after the corrections, the associations remained significant for the rs907094 allele (corrected $p=0.040$) and the rs12601930 genotype (corrected $p=0.000588$). We, however, could not confirm these

Table 2-1
Genotype and allele frequency of *PPP1R1B* in bipolar disorder and controls (first sample set)

Gene symbol	SNP ID (M/m)	Method of genotyping	Genotype[a] BP[b]			CON[b]			p value	Armitage's p value	Allele BP		CON		p value	Global p value[c]
PPP1R1B	rs879606 (G/A)	RFLP	G/G 87 (30%)	G/A 146 (50%)	A/A 61 (21%)	G/G 58 (32%)	G/A 91 (49%)	A/A 35 (19%)	0.857	0.580	G 320 (54%)	A 268 (46%)	G 207 (56%)	A 161 (44%)	0.306	0.030
	rs12601930 (C/T)	real time PCR	C/C 169 (69%)	C/T 57 (23%)	T/T 18 (7%)	C/C 240 (63%)	C/T 134 (35%)	T/T 8 (2%)	0.000147	0.804	C 395 (81%)	T 3 (19%)	C 614 (80%)	T 150 (20%)	0.802	
	rs907094 (T/C)	real time PCR	T/T 63 (23%)	T/C 202 (54%)	C/C 71 (19%)	T/T 114 (30%)	T/C 193 (50%)	C/C 77 (20%)	0.036	0.010	T 267 (48%)	C 293 (52%)	T 421 (55%)	C 347 (45%)	0.010	
	rs3764352 (A/G)	RFLP	A/A 64 (22%)	A/G 170 (58%)	G/G 58 (20%)	A/A 104 (28%)	A/G 195 (52%)	G/G 79 (21%)	0.176	0.385	A 298 (51%)	G 286 (49%)	A 403 (53%)	G 353 (47%)	0.407	

[a]M: major allele, m: minor allele [b]BP: bipolar disorder, CON: control [c]global p value: haplotypic analysis.

Table 2-2
Genotype and allele frequency of *PPP1R1B* in bipolar disorder and controls (second sample set)

Gene symbol	SNP ID (M/m)	Method of genotyping	Genotype[a] BP[b]			CON[b]			p value	Armitage's p value	Allele BP		CON		p value	Global p value[c]
PPP1R1B	Rs12601930 (C/T)	real time PCR	C/C 232 (63%)	C/T 119 (33%)	T/T 15 (4%)	C/C 248 (68%)	C/T 105 (29%)	T/T 12 (3%)	0.419	0.192	C 583 (80%)	T 149 (20%)	C 601 (82%)	T 129 (18%)	0.191	0.307
	Rs907094 (T/C)	real time PCR	T/T 102 (28%)	T/C 190 (53%)	C/C 70 (19%)	T/T 107 (29%)	T/C 180 (49%)	C/C 81 (22%)	0.565	0.731	T 394 (54%)	C 330 (46%)	T 394 (54%)	C 342 (46%)	0.734	

[a]M: major allele, m: minor allele [b]BP: bipolar disorder, CON: control [c]global p value: haplotypic analysis.

Table 3-1
PPP1R1B copy number variations detected by qPCR[a] (first sample set)

	SCZ[b] (n=24)	BP[b] (n=24)	CON[b] (n=24)
Relative quantity	0.81 ± 0.14	0.87 ± 0.10	1.11 ± 0.34
Decrease	6	3	2
Normal	17	21	15
Increase	1	0	7

[a]qPCR: quantitative real-time PCR.
[b]SCZ: schizophrenia, BP: bipolar disorder, CON: control.

results in a second independent sample set as shown in Table 2-2. When the two sample sets were merged, power analysis showed the level for detecting association was higher than 80% for bipolar disorder at the genotype relative risk of 1.24 to 1.39 under a multiplicative model of inheritance.

DNA copy number analysis revealed aberrations in the copy number in the schizophrenic patients (increase=1 patient, decrease=6), the bipolar patients (decrease=3), and the control subjects (increase=7, decrease=2; Table 3-1). In the second independent sample set, we observed aberrations in the schizophrenic patients (increase=2, decrease=1), the bipolar patients (increase=2, decrease=1), and the control subjects (increase=2, decrease=5; Table 3-2). All analyses were performed in duplicate.

4. Discussion

According to the common disease-common variants hypothesis (Chakravarti, 1999), the present study showed that *PPP1R1B* was unlikely to be related to be the development of schizophrenia and bipolar disorder in Japanese patients. These results were consistent with a recently published study that examined Chinese patients (Li et al., 2006).

The SNPs used in the association analysis, which covered the entire gene, included all of the common SNPs (more than 5% frequency) listed in the dbSNP database; therefore, it is unlikely that there are other common variants related to these disorders. Because we did not perform mutation screening of this gene, however, the possibility that rare variants could be causal to the development of these disorders cannot be excluded. The GRR value calculated using power analysis was appropriate when compared to other promising candidate genes for schizophrenia (Schwab et al., 2005; Schwab et al., 2003; Shifman et al., 2002).

Recently, Meyer-Lindenberg et al. (2007) tested for an association in schizophrenia with SNPs in this gene using a relatively small sample of Caucasian families, and found a strong association with rs879606 and mild association with two other SNPs (rs3764352 and rs3794712). Two of these three SNPs (rs879606 and rs3764352), however, did not show any significant association in our samples, suggesting that ethnic differences might play a role in these associations.

rs3794712 was not further pursued for two reasons. *Firstly,* this SNP is not validated and the frequency of this SNP is not reported in the dbSNP database. *Secondly,* this SNP is unlikely to affect the function of this gene considering its genomic position in the middle of an intron and the results from *in silico* analysis (RegRNA; http://regrna.mbc.nctu.edu.tw/index.html).

In the present study, we found that copy number differences in the region that includes the SNPs deviated from the HWE and were significantly associated with bipolar disorder (Tables 3-1 and 3-2), suggesting that the presence of copy number alterations gives rise to the deviation from the HWE due to a high frequency of heterozygotes. Therefore, caution must be taken when interpreting results from SNP analysis. These results, however, must be regarded as preliminary because we did not observe a definite association between copy number differences and the disorders. Because accumulating evidence has highlighted that CNVs are observed in many chromosome regions, *including 17q12* (Sharp et al., 2006), and copy number alterations can affect gene expression (Redon et al., 2006), further investigations of the CNVs as well as the SNPs may be an effective complementary approach to elucidate the genetic risk factors underlying the complex phenotypes associated with psychiatric disorders, including schizophrenia and bipolar disorder (Lee and Lupski, 2006).

A couple of limitations should be considered in the present study. Firstly, we observed a strong association between rs12601930 and bipolar disorder in the first sample set even after a Bonferroni correction (corrected genotype $p=0.000588$); the genotype frequency of the minor allele homozygotes (TT) in the control subjects, however, was only 2% (Table 2-1). This low frequency may have resulted in a false-positive association. When we used a Cochran–Armitage trend test (Balding, 2006)

Table 3-2
PPP1R1B copy number variations detected by qPCR[a] (second sample set)

	SCZ[b] (n=24)	BP[b] (n=24)	CON[b] (n=24)
Relative quantity	0.95 ± 0.10	0.98 ± 0.14	1.03 ± 0.27
Decrease	2	2	2
Normal	69	69	65
Increase	1	1	5

[a]qPCR: quantitative real-time PCR.
[b]SCZ: schizophrenia, BP: bipolar disorder, CON: control.

to correct the observed association with rs12601930, which deviated from the HWE, there was no significance in the genotype frequency (Armitage's $p=0.804$). Thus, the possibility of a type I error derived from an insufficient sample size should be considered. Secondly, although the method used for copy number analysis has been widely used and is thought to be highly reliable due to the consistency of results obtained in our duplicate experiments, supplementary methods, such as Southern blotting, fluorescence in situ hybridization, or array comparative genomic hybridization, would have provided further confirmation of our results. Finally, the male/female ratios and average ages were not completely consistent between the schizophrenic patients and the control subjects. Based on results from an exploratory analysis using a logistic regression model, however, these variables do not appear to contribute to the results obtained in the present association study (data not shown).

In conclusion, our findings suggest that SNPs within *PPP1R1B* do not elevate the risk for either schizophrenia or bipolar disorder in the Japanese population. Further functional analysis of the CNVs and association studies using other endophenotypes including cognitive function should be needed to clarify the exact role of this gene in the pathophysiology of these disorders.

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Contributors

Author Akira Yoshimi, Nagahide Takahashi, and Shinichi Saito designed the study and wrote the protocol. Author Norio Ozaki and Yukihiro Noda performed the literature searches and analyses. Author Akira Yoshimi wrote the first draft of the manuscript and Nagahide Takahashi and Shinichi Saito revised it. All of the authors contributed to and have approved the final version of the manuscript.

Conflict of interest

All authors declare that they have no conflicts of interests.

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References

Albert, K.A., Hemmings Jr., H.C., Adamo, A.I., Potkin, S.G., Akbarian, S., Sandman, C.A., Cotman, C.W., Bunney Jr., W.E., Greengard, P., 2002. Evidence for decreased DARPP-32 in the prefrontal cortex of patients with schizophrenia. Arch. Gen. Psychiatry 59 (8), 705–712.

Balding, D.J., 2006. A tutorial on statistical methods for population association studies. Nature reviews 7 (10), 781–791.

Beneyto, M., Kristiansen, L.V., Oni-Orisan, A., McCullumsmith, R.E., Meador-Woodruff, J.H., 2007. Abnormal glutamate receptor expression in the medial temporal lobe in schizophrenia and mood disorders. Neuropsychopharmacology.

Cardno, A.G., Holmans, P.A., Rees, M.I., Jones, L.A., McCarthy, G.M., Hamshere, M.L., Williams, N.M., Norton, N., Williams, H.J., Fenton, I., Murphy, K.C., Sanders, R.D., Gray, M.Y., O'Donovan, M.C., McGuffin, P., Owen, M.J., 2001. A genomewide linkage study of age at onset in schizophrenia. Am. J. Med. Genet. 105 (5), 439–445.

Catapano, L.A., Manji, H.K., 2007. G protein-coupled receptors in major psychiatric disorders. Biochim. Biophys. Acta. 1768 (4), 976–993.

Chakravarti, A., 1999. Population genetics-making sense out of sequence. Nat. Genet. 21 (1 Suppl), 56–60.

de Bakker, P.I., Yelensky, R., Pe'er, I., Gabriel, S.B., Daly, M.J., Altshuler, D., 2005. Efficiency and power in genetic association studies. Nat. Genet. 37 (11), 1217–1223.

Dick, D.M., Foroud, T., Flury, L., Bowman, E.S., Miller, M.J., Rau, N.L., Moe, P.R., Samavedy, N., El-Mallakh, R., Manji, H., Glitz, D.A., Meyer, E.T., Smiley, C., Hahn, R., Widmark, C., McKinney, R., Sutton, L., Ballas, C., Grice, D., Berrettini, W., Byerley, W., Coryell, W., DePaulo, R., MacKinnon, D.F., Gershon, E.S., Kelsoe, J.R., McMahon, F.J., McInnis, M., Murphy, D.L., Reich, T., Scheftner, W., Nurnberger Jr., J.I., 2003. Genomewide linkage analyses of bipolar disorder: a new sample of 250 pedigrees from the National Institute of Mental Health Genetics Initiative. Am. J. Hum. Genet. 73 (1), 107–114.

Dudbridge, F., 2003. Pedigree disequilibrium tests for multilocus haplotypes. Genet. Epidemiol. 25 (2), 115–121.

Fienberg, A.A., Hiroi, N., Mermelstein, P.G., Song, W., Snyder, G.L., Nishi, A., Cheramy, A., O'Callaghan, J.P., Miller, D.B., Cole, D.G., Corbett, R., Haile, C.N., Cooper, D.C., Onn, S.P., Grace, A.A., Ouimet, C.C., White, F.J., Hyman, S.E., Surmeier, D.J., Girault, J., Nestler, E.J., Greengard, P., 1998. DARPP-32: regulator of the efficacy of dopaminergic neurotransmission. Science 281 (5378), 838–842.

Ikeda, M., Iwata, N., Suzuki, T., Kitajima, T., Yamanouchi, Y., Kinoshita, Y., Inada, T., Ozaki, N., 2005. No association of haplotype-tagging SNPs in TRAR4 with schizophrenia in Japanese patients. Schizophr. Res. 78 (2-3), 127–130.

Lee, J.A., Lupski, J.R., 2006. Genomic rearrangements and gene copy-number alterations as a cause of nervous system disorders. Neuron 52 (1), 103–121.

Li, C.H., Liao, H.M., Hung, T.W., Chen, C.H., 2006. Mutation analysis of DARPP-32 as a candidate gene for schizophrenia. Schizophr. Res. 87 (1–3), 1–5.

Meyer-Lindenberg, A., Straub, R.E., Lipska, B.K., Verchinski, B.A., Goldberg, T., Callicott, J.H., Egan, M.F., Huffaker, S.S., Mattay,

V.S., Kolachana, B., Kleinman, J.E., Weinberger, D.R., 2007. Genetic evidence implicating DARPP-32 in human frontostriatal structure, function, and cognition. J. Clin. Invest. 117 (3), 672–682.

Murray, R.M., Sham, P., Van Os, J., Zanelli, J., Cannon, M., McDonald, C., 2004. A developmental model for similarities and dissimilarities between schizophrenia and bipolar disorder. Schizophr. Res. 71 (2–3), 405–416.

Nishi, A., Watanabe, Y., Higashi, H., Tanaka, M., Nairn, A.C., Greengard, P., 2005. Glutamate regulation of DARPP-32 phosphorylation in neostriatal neurons involves activation of multiple signaling cascades. Proc. Natl. Acad. Sci. U.S.A. 102 (4), 1199–1204.

Purcell, S., Cherny, S.S., Sham, P.C., 2003. Genetic power calculator: design of linkage and association genetic mapping studies of complex traits. Bioinformatics 19 (1), 149–150.

Redon, R., Ishikawa, S., Fitch, K.R., Feuk, L., Perry, G.H., Andrews, T.D., Fiegler, H., Shapero, M.H., Carson, A.R., Chen, W., Cho, E.K., Dallaire, S., Freeman, J.L., Gonzalez, J.R., Gratacos, M., Huang, J., Kalaitzopoulos, D., Komura, D., MacDonald, J.R., Marshall, C.R., Mei, R., Montgomery, L., Nishimura, K., Okamura, K., Shen, F., Somerville, M.J., Tchinda, J., Valsesia, A., Woodwark, C., Yang, F., Zhang, J., Zerjal, T., Armengol, L., Conrad, D.F., Estivill, X., Tyler-Smith, C., Carter, N.P., Aburatani, H., Lee, C., Jones, K.W., Scherer, S.W., Hurles, M.E., 2006. Global variation in copy number in the human genome. Nature 444 (7118), 444–454.

Schwab, S.G., Hoefgen, B., Hanses, C., Hassenbach, M.B., Albus, M., Lerer, B., Trixler, M., Maier, W., Wildenauer, D.B., 2005. Further evidence for association of variants in the AKT1 gene with schizophrenia in a sample of European sib-pair families. Biol. Psychiatry 58 (6), 446–450.

Schwab, S.G., Knapp, M., Mondabon, S., Hallmayer, J., Borrmann-Hassenbach, M., Albus, M., Lerer, B., Rietschel, M., Trixler, M., Maier, W., Wildenauer, D.B., 2003. Support for association of schizophrenia with genetic variation in the 6p22.3 gene, dysbindin, in sib-pair families with linkage and in an additional sample of triad families. Am. J. Hum. Genet. 72 (1), 185–190.

Sharp, A.J., Hansen, S., Selzer, R.R., Cheng, Z., Regan, R., Hurst, J.A., Stewart, H., Price, S.M., Blair, E., Hennekam, R.C., Fitzpatrick, C.A., Segraves, R., Richmond, T.A., Guiver, C., Albertson, D.G., Pinkel, D., Eis, P.S., Schwartz, S., Knight, S.J., Eichler, E.E., 2006. Discovery of previously unidentified genomic disorders from the duplication architecture of the human genome. Nat. Genet. 38 (9), 1038–1042.

Shifman, S., Bronstein, M., Sternfeld, M., Pisante-Shalom, A., Lev-Lehman, E., Weizman, A., Reznik, I., Spivak, B., Grisaru, N., Karp, L., Schiffer, R., Kotler, M., Strous, R.D., Swartz-Vanetik, M., Knobler, H.Y., Shinar, E., Beckmann, J.S., Yakir, B., Risch, N., Zak, N.B., Darvasi, A., 2002. A highly significant association between a COMT haplotype and schizophrenia. Am. J. Hum. Genet. 71 (6), 1296–1302.

Svenningsson, P., Tzavara, E.T., Carruthers, R., Rachleff, I., Wattler, S., Nehls, M., McKinzie, D.L., Fienberg, A.A., Nomikos, G.G., Greengard, P., 2003. Diverse psychotomimetics act through a common signaling pathway. Science 302 (5649), 1412–1415.

Svenningsson, P., Tzavara, E.T., Witkin, J.M., Fienberg, A.A., Nomikos, G.G., Greengard, P., 2002. Involvement of striatal and extrastriatal DARPP-32 in biochemical and behavioral effects of fluoxetine (Prozac). Proc. Natl. Acad. Sci. U. S. A. 99 (5), 3182–3187.

Wilson, G.M., Flibotte, S., Chopra, V., Melnyk, B.L., Honer, W.G., Holt, R.A., 2006. DNA copy-number analysis in bipolar disorder and schizophrenia reveals aberrations in genes involved in glutamate signaling. Hum. Mol. Genet. 15 (5), 743–749.

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Failure to replicate the association between *NRG1* and schizophrenia using Japanese large sample

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Abstract

Systematic linkage disequilibrium (LD) mapping of 8p12–21 in the Icelandic population identified neuregulin 1 (*NRG1*) as a prime candidate gene for schizophrenia. However, results of replication studies have been inconsistent, and no large sample analyses have been reported. Therefore, we designed this study with the aim of assessing this putative association between schizophrenia and *NRG1* (especially HAP_{ICE} region and exon region) using a gene-based association approach in the Japanese population.

This study was a two-stage association analysis with a different panel of samples, in which the significant association found in the first-set screening samples (1126 cases and 1022 controls) was further assessed in the confirmation samples (1262 cases and 1172 controls, and 166 trio samples). In the first-set scan, 60 SNPs (49 tagging SNPs from HapMap database, four SNPs from other papers, and seven SNPs detected in the mutation scan) were examined.

One haplotype showed a significant association in the first-set screening samples (Global *P*-value = 0.0244, uncorrected). However, we could not replicate this association in the following independent confirmation samples. Moreover, we could not find sufficient evidence for association of the haplotype identified as being significant in the first-set samples by imputing ungenotyped SNPs from HapMap database.

Abbreviations: NRG1, neuregulin 1; SNP, single nucleotide polymorphism; GGF2, glial growth factor 2; LD, linkage disequilibrium; dHPLC, denaturing high performance liquid chromatography; MAF, minor allele frequency; TDT, transmission disequilibrium test; UTR, untranslated region.

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These results indicate that the positionally and functionally attractive regions of *NRG1* are unlikely to contribute to susceptibility to schizophrenia in the Japanese population. Moreover, the nature of our results support that two-stage analysis with large sample size is appropriate to examine the susceptibility genes for common diseases.
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Keywords: Schizophrenia; Neuregulin 1; Association study; False positive; Linkage disequilibrium

1. Introduction

Schizophrenia is a common psychiatric disorder with a lifetime prevalence of 1% worldwide. Family, twin and adoption studies show conclusive evidence of a substantial genetic component in this disorder. Progress towards detecting these genetic elements is now being made (Harrison and Weinberger, 2005).

The neuregulin 1 gene (*NRG1*) was first reported to be a prime candidate gene for schizophrenia in the Icelandic population (Stefansson et al., 2002). The significant association of a haplotype was detected in the 5′-region of glial growth factor 2 (GGF2) isoforms, and this at-risk haplotype, consisting of five single nucleotide polymorphisms (SNPs) and two microsatellites, was named as HAP_{ICE}. Several subsequent studies provided the following evidence to support this association with schizophrenia.

Firstly, the location of this gene corresponds to the linkage regions for schizophrenia (8p12–21, OMIM: SCZ5), which were identified by recent meta-analyses of genome-wide linkage studies (Badner and Gershon 2002; Lewis et al., 2003). Secondly, recent evidence suggests that mutation within the *NRG1* region might give rise to functional alterations that are in line with the neurodevelopmental hypothesis and glutamate/GABA hypothesis of schizophrenia (Corfas et al., 2004).

Thirdly, several independent association studies have replicated the original significant association found by Stefansson et al. (2002). However, the results of replication studies using the identical number or fewer sets of markers have been inconsistent. Thus, while some research groups did not report any association (Iwata et al., 2004), other studies showed a positive association but showed different 'at-risk' haplotypes to be associated with schizophrenia (Harrison and Law, 2006).

These inconsistent results could stem from the possibility that *NRG1* is not involved in the etiology of schizophrenia in all populations. However, this inconsistency could be a consequence of the unique structure of the human genome. In other words, differences in linkage disequilibrium (LD) among populations may also be responsible for the differences in the results, and the negative findings may only indicate a failure to reflect the actual predisposing variants due to the differences in populations.

Therefore, gene-wide (or region-wide) replication analysis based on LD pattern within the *NRG1* region is essential to detect an association in a certain population setting (Neale and Sham, 2004). In such analyses, particular attention should be paid to selection of genetic variants which adequately reflect the LD background in the targeted population (e.g. tagging SNPs).

Although the above-mentioned LD-based association analysis is based on the common disease–common variant hypothesis, one study reported an association between *NRG1* and schizophrenia from the standpoint of the common disease–rare variant hypothesis (Walss-Bass et al., 2006). The authors scanned the whole exon region, detected a non-synonymous SNP in exon 11, and showed a significant association of this SNP with schizophrenia. Detection of rare but potent functional variants relies on large mutation scan samples; however, such rare variants may also differ among populations (Pritchard, 2001).

Thus, in this study, we first focused on two attractive regions: the 5′ regions of GGF2, where the original study showed the association (henceforth referred to as 'HAP_{ICE} region') and the exon region (henceforth referred to as 'exon region'). In the exon region, prior to association analysis of tagging SNPs, we performed a mutation scan in order to detect the existence of possible potent functional variants in the ethnic samples. In addition, this study was a two-stage association analysis with a different panel of samples, in which the significant association in the first-set screening samples (1126 cases and 1022 controls) was further assessed in confirmation samples (1262 cases, 1172 controls, and 166 trio samples). This approach was adopted in order to avoid the possibility of type I or type II error.

2. Methods and materials

2.1. Subjects

Two independent sample sets were used in this study. For the first-set screening analysis, 1126 patients with schizophrenia (627 male and 499 female; mean age± standard deviation (SD) 47.0±15.3 years) and 1022

healthy controls (530 male and 492 female; 38.8±14.5 years) were examined. Confirmation analysis was conducted with three samples consisting of: (a) 1262 patients with schizophrenia (662 male and 600 female; 49.1±14.5 years) (b) 1172 controls (576 male and 596 female; 41.7±14.3 years), and (c) 166 family trios samples (of the patients, 91 male and 75 female; 30.0±8.3 years).

The subjects for mutation search were 96 patients with schizophrenia. These subjects were also included in the first-set samples. 385 cases and 336 controls in the first-set samples, and 349 cases (including 84 cases from family samples) and 424 controls in confirmation samples are identical to those in our previous report (Iwata et al., 2004) and Fukui et al.'s (2006) report, respectively.

Characterization details and psychiatric assessment of these subjects were as follows. The patients were diagnosed according to DSM-IV criteria consensus of at least two experienced psychiatrists on the basis of unstructured interviews and review of medical records. All healthy controls were also psychiatrically screened based on unstructured interviews. All subjects were ethnically Japanese.

After the study had been described to subjects, written informed consent was requested from each. This study was approved by the ethics committees at Fujita Health University, Teikyo University, Okayama University, Osaka University, Niigata University and Nagoya University Graduate School of Medicine.

2.2. Mutation scan

We performed denaturing high performance liquid chromatography (dHPLC) analysis, details of which can be seen in a previous paper (Ikeda et al., 2005). Primer sequences were designed in accordance with another report (Walss-Bass et al., 2006).

2.3. Tagging SNP selection

We included the three signal SNPs (SNP8NRG221533, SNP8NRG241930 and SNP8NRG243177) from the report of Stefansson et al. (2002) (we excluded SNP8NRG221132 and SNP8NRG433E1006 from the first-set analysis due to low minor allele frequencies (MAFs) in the Japanese population), one positive SNP from the report of Walss-Bass et al. (2006), and SNPs we detected in the mutation scan. Next we consulted the HapMap database (release#19, population: Japanese in Tokyo (JPT), MAF: more than 0.05). In this step, we determined the boundaries of the 'HAP_{ICE} regions' that cover 5′ regions including 19,425 bp and 155,564 bp downstream (3′) from the significant SNPs (SNP8NRG221132 and SNP8NRG433E1006, respectively) in Stefansson's report (Table 1 and Supplementary Fig. 1) (Stefansson et al., 2002), and of the 'exon regions' that cover 5′ regions including 120,576 bp from the first exon and 3510 bp downstream 3′ from the last exon (GenBank accession No. NT_007995: Table 2 and Supplementary Fig. 2). Then fifteen and thirty-four 'tagging SNPs' for the HAP_{ICE} regions and exon regions, respectively were selected with the criterion of an r^2 threshold greater than 0.8 in 'Aggressive tagging: use 2- and 3-markers haplotypes' mode of the 'Tagger' program (de Bakker et al., 2005), a function of HAPLOVIEW software (Barrett et al., 2005).

2.4. SNP genotyping

All SNPs were genotyped by TaqMan assay (Applied Biosystems Japan Ltd, Tokyo).

The genotyping of C#5, C#6, C#7 (which were positive SNPs in the first-set screening analysis) was done with 768 randomly selected samples (384 cases and 384 control subjects) with direct sequencing to check for genotyping error. Detailed information including primer sequences of custom TaqMan SNP genotyping assays can be seen in Supplementary Tables 1 and 2.

Table 1
First-set case control analysis of HAP_{ICE} region

Markers	SNP ID	*P*-values		
		1-window	2-windows	3-windows
HAP_{ICE}#1	rs12674974	.0794	.181	
HAP_{ICE}#2	rs4513929	.846	.384	.196
HAP_{ICE}#3	SNP8NRG221533	.188	.397	.620
HAP_{ICE}#4	rs10096573	.200	.414	.462
HAP_{ICE}#5	rs4733263	.310	.616	.267
HAP_{ICE}#6	rs4733263	.274	.399	.578
HAP_{ICE}#7	SNP8NRG241930	.724	.113	.326
HAP_{ICE}#8	SNP8NRG243177	.288	.520	.492
HAP_{ICE}#9	rs4733267	.769	.889	.190
HAP_{ICE}#10	rs13277456	.862	.736	.847
HAP_{ICE}#11	rs13274954	.457	.670	.255
HAP_{ICE}#12	rs12677942	.312	.271	.128
HAP_{ICE}#13	rs4403369	.0803	.268	.548
HAP_{ICE}#14	rs4566990	.625	.628	.525
HAP_{ICE}#15	rs13270788	.541	.730	.699
HAP_{ICE}#16	rs1503491	.813	.866	.0960
HAP_{ICE}#17	rs2202262	.704	.324	.0653
HAP_{ICE}#18	rs10087212	.682		
HAP_{ICE}#4–#5		.414		
HAP_{ICE}#14–#16		.247		
HAP_{ICE}#15–#16		.730		

Table 2
First-set case control analysis of exon region

Markers	SNP ID	*P*-values[a]		
		1-window	2-windows	3-windows
C#1	rs10503915	.116	.0603	
C#2	rs7016691	.231	.371	.349
C#3	rs11782671	.472	.474	.296
C#4	rs10103930	.168	.322	.508
C#5	rs10503917	.699	.628	.0935
C#6	rs10107065	.765	.138	**.0244**
C#7	rs6468118	.138	.154	.174
C#8	rs7000590	.0939	.107	.158
MS1	rs7820838	.110	.142	.181
MS2	rs7834206	.149	.0879	.145
C#9	rs4236709	.0786	.187	.352
C#10	rs13260545	.0994	.248	.403
C#11	rs4316112	.948	.144	.0984
C#12	rs2439305	.196	.130	.132
C#13	rs7826814	.715	.851	.129
C#14	rs2466064	.690	.313	.436
MS3	rs3924999	.162	.113	.0699
C#15	rs10954864	.803	.969	.602
C#16	rs2439281	.965	.0725	.301
C#17	rs9642729	.0680	.0988	.137
C#18	rs12547858	.0801	.457	.523
C#19	rs10098373	.801	.835	.654
C#20	rs10095694	.380	.727	.872
MS4	rs3735774	.762	.727	.718
C#21	rs2466058	.372	.526	.587
C#22	rs2466052	.379	.286	.509
C#23	rs2466046	.187	.372	.431
C#24	rs10503923	.546	.473	.203
C#25	rs2466084	.310	.551	.197
C#26	rs2976515	.253	.654	.563
C#27	rs4445183	.702	.484	.500
C#28	rs2919377	.151	.341	.455
C#29	rs2919375	.819	.222	.182
MS5	rs3735776	.740	.758	.129
C#30	rs7007436	.711	.815	.866
C#31	rs3757934	.758	.421	.562
MS7	rs4733376	.379	.336	.357
C#32	rs4360253	.357	.893	.789
C#33	rs7005288	.864	.812	.738
C#34	rs6992642	.569		
MS6 (C#24–#30)[b]	rs17731664	.772		
C#5–#11–#14		1.00		
C#5–#14		.180		
C#16–#27		.751		
C#23–#26–#28		.245		

[a] Bold number represents significant *P*-value.
[b] MS6 could be represented by the haplotypes constructed by C#24–30.

2.5. Statistical methods for conventional association analysis

In the case–control samples, the marker–trait association was evaluated with the χ^2 test in allele- and genotype-wise analyses. Haplotype frequencies were estimated in a 2- to 3-marker sliding window fashion by EM algorithm and Log likelihood ratio tests were performed for Global *P*-values with COCAPHASE program version 3.06 (Dudbridge, 2003). In the family samples, the transmission disequilibrium test (TDT) and 3-marker haplotype analyses were performed with the TDTPHASE program version 3.06 (Dudbridge, 2003). In these haplotype-wise analyses, rare haplotypes (less than 0.05) of cases and controls were excluded from the association analysis in order to provide greater sensitivity and accuracy.

The significance level was set at $P<0.05$.

2.6. Imputation of ungenotyped SNPs

Our conventional haplotype-wise analysis was done in a sliding window fashion, since our selection for tagging SNPs was not based on the haplotype block concept. Although this type of haplotype-wise analysis does not adapt to the degree of LD, so that it is unclear which markers should be considered jointly, it results in a higher level of statistical power since it can reflect unknown SNPs that were not included in the analysis. Considering this, we included a recently developed method, imputation, to test for any SNPs that reflect the significant haplotypes (Marchini et al., 2007). The IMPUTE program imputes the genotypic distribution of un-observed SNPs using observed SNP information (60 SNPs used in the screening scan) and the HapMap database (fine-scale recombination map, haplotype for JPT/CHP).

The targeted region for imputation was limited to within known recombination hot spots, because our data targeted only the HAP_{ICE} and exon regions.

After imputation, we applied a Bayesian test with an additive model to assess the association using SNPTEST software (Marchini et al., 2007). Default values were used in all settings needed in IMPUTE and SNPTEST (e.g. effective population size for JPT/CHP, buffer, call threshold for calling genotyped SNPs and number of samples of genotypes that should be used for Bayesian tests).

Table 3
Individual haplotype analyses from significant Global *P*-values in first-set samples

	haplotypes	Case Freq (%)	Con Freq (%)	*P*-value	Global *P*-value
C#5–6–7	1–1–1	9.36	11.8	.0104	.0244
	1–1–2	15.6	13.6	.0896	
	1–2–2	65.8	65.5	.886	
	2–1–1	7.21	6.27	.300	

2.7. Power calculation

Power calculation was performed with a web-based statistical program, Genetic Power Calculator (Purcell et al., 2003). Power was estimated under a multiplicative model of inheritance, assuming the disease prevalence to be 1% and the population susceptibility allele frequencies to be the values observed in control samples.

3. Results

3.1. Mutation scan and first-set association analysis

We detected seven SNPs through dHPLC analysis of the exon region (MS1–7: Table 2). One of them, MS3 (rs3924999), is a non-synonymous SNP (Gly38Arg) and had shown a significant association in the Chinese population (Yang et al., 2003). The other SNPs were located in an untranslated region (UTR) or branch site, and may therefore have a functional effect (Table 2).

Next, 49 SNPs and 7 haplotypes were selected as Tagging SNPs from the HapMap database. These SNPs are located in the HAP_{ICE}- and coding regions based on the HapMap database (Tables 1 and 2).

Consequently, by involving 11 SNPs (the 7 SNPs we detected and 4 SNPs reported in other papers (Stefansson et al., 2002; Walss-Bass et al., 2006)), a total of 60 SNPs were genotyped in the first-set screening samples (however, since we were unable to design a genotyping method for one SNP that we detected (MS6) by TaqMan Assay by Design (Applied Biosystems), we determined the genotype distribution of some samples (192 cases and 192 controls) using a direct sequencing method. With these samples we confirmed that MS6 could be represented by the haplotypes constructed by C#24–30 in LD evaluation.).

The SNP for which significance was shown in the report of Walss-Bass et al. (2006) was not polymorphic in our samples.

Allele- and genotype-wise analyses did not show association either the HAP_{ICE} region or the exon region. In this haplotype-wise analysis, 3-marker haplotypes of C#5–6–7 were associated with schizophrenia (Global P-value=0.0244, uncorrected: Tables 1, 2 and 3, Supplementary Tables 3 and 4). The genotyping of C#5, C#6, C#7 in a subset of the screening samples was re-confirmed by direct sequencing, and the results were perfectly identical to those shown by TaqMan assay. Hence, we speculate that it was unlikely that genotyping error had occurred.

3.2. Imputation of ungenotyped SNP for first-set samples

Data for ungenotyped SNPs could not provide sufficient evidence for association in either region (Fig. 1). In particular, the weights of evidence for the regions near the significant haplotypes in first-set samples were less than one. Since weights of evidence of at least four are required for evidence for association

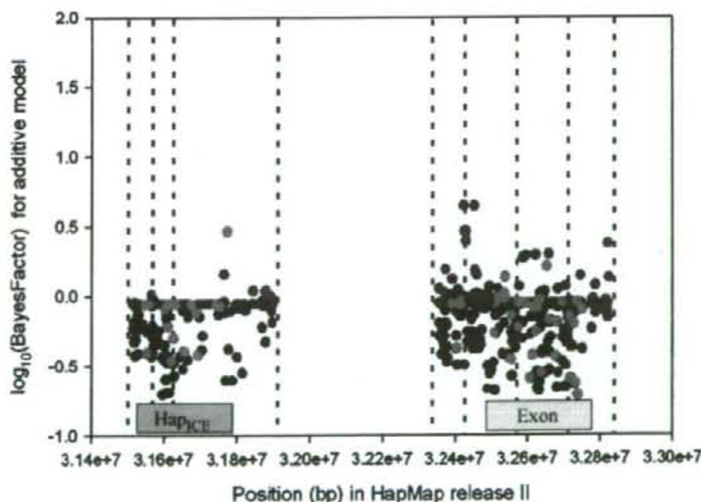

Fig. 1. Results of imputing SNP in the *NRG1* gene. The weights of evidence were calculated using imputed genotypes (red circles) and observed genotypes (black circles). Data from SNPs that constructed the significant haplotype in the first-set samples are shown in blue circles. Dotted lines indicate the estimated hot spots from the HapMap database. The SNP position from the HapMap release II database is plotted on the X axis.

Table 4
Confirmation analysis of significant haplotypes from first-set analysis

Samples	SNPID	1-window	2-windows	3-windows
Case–control	C#5	.408		
	C#6	.362	.101	.120
	C#7	.371	.601	
Family samples	C#5	.107		
	C#6	.964	.323	.505
	C#7	.499	.846	
Combined samples	C#5	.976		
	C#6	.389	.591	.478
	C#7	.801	.303	

(if 1000 SNPs of 10,000,000 common human SNPs might be associated with a disease, we may assign a prior odds of association of 1/10,000. Therefore, a Bayes factor more than 10,000 (or $\log_{10}$ [Bayes factor] more than 4) is required (Balding, 2006)). Thus, these results indicate a low probability for association in our sample.

3.3. *Confirmation analysis of the positive haplotypes using different case–control samples and family samples*

To confirm the significance of exon region C#5–6–7 in the first-set samples, we conducted a confirmation analysis using independent case–control samples and family samples. In these analyses, we could not replicate this association. To increase the power, we combined samples (first-set and confirmation samples) but again we could not detect an association in this explorative analysis (Table 4).

4. Discussion

In the present study, using three large and independent samples, our data did not provide sufficient evidence for associations between tagging SNPs in the HAP_{ICE} and exon regions of *NRG1* and schizophrenia in the Japanese population.

We could not replicate previous reports for the HAP_{ICE} region (Stefansson et al., 2002; Stefansson et al., 2003); however, the results of this study are in concordance with our previous replication study in the Japanese population (schizophrenia=607, controls=515) (Iwata et al., 2004). Another study (Fukui et al., 2006), however, examined independent Japanese samples (belonging to one-third of confirmation case–control samples) and reported a positive association. Specifically, that study reported a significant association of haplotypes constructed by three core SNPs from Stefansson et al. (SNP8NRG221533 (HAP_{ICE}#3), SNP8NRG241930 (HAP_{ICE}#7) and SNP8NRG243177 (HAP_{ICE}#8)), and one more intronic SNP (rs1081062), as well as a trend for association of rs1081062. Since our tagging SNPs could not involve this SNP (rs1081062), we found by consulting the latest HapMap database (release#21a) that rs1081062 is tagged by rs13274954 (HAP_{ICE}#11); moreover, neither HAP_{ICE}#10 nor its haplotypes (HAP_{ICE}#3–7–8–11) were associated with schizophrenia (Global P-value= 0.540). Therefore, the aforementioned positive report could have been the result of type I error due to inadequate sample size (schizophrenia=349, controls=424) (Fukui et al., 2006). Or, as the authors speculated (Fukui et al., 2006), the different clinical backgrounds (e.g. genetic loading) in each sample could have led to inconsistent results. In this regard, a recent study reported that *DAOA/G30*, which is also a strong candidate gene for schizophrenia, influences susceptibility to the symptomatology of psychiatric disorders including schizophrenia and bipolar disorder, but not to diagnosis itself (Williams et al., 2006).

In the coding region, our results indicated the importance of controlling inflation of the type I error rate due to multiple testing, when a significant association is obtained in an analysis that involves several markers. In this study we found significant associations only from haplotype-wise analysis, not from allele- or genotype-wise analysis. It is generally accepted that a haplotype-wise analysis gives high power. At the same time, haplotype-wise analysis, especially multi-marker analysis or sliding-window analysis, tends to increase the chance of false positive results, since numerous hypotheses are examined. Bonferroni correction is typically used for solving multiple testing problems; however, since markers are not independent due to the existence of LD, Bonferroni correction is thought to be too conservative.

Therefore, we adopted two methods to validate the observed association; firstly, we imputed ungenotyped SNPs that might reflect a significant haplotype based on observations including our genotypic distribution of tagging SNPs and LD structure from the HapMap database. However, our simulation suggests that results for ungenotyped SNPs do not provide sufficient evidence for association. In other words, there was no SNP which could reflect a significant haplotype in the current data in HapMap release II. Secondly, we examined independent sets of samples for which a significant association was obtained in the initial screening analysis. We considered this to be the best strategy at present; however, the former significance of the exon region haplotype could not be replicated though independent case–control and family trios samples.

It is unlikely that negative results are due to type II error since a large sample size was used in this study; moreover, power analyses showed that the power was more than 80% when genotype relative risk (GRR) was set at 1.2–1.65 and 1.6–3.1 for confirmation case–