Table 2
Second-set confirmation analysis of Marker5

	Phenotype[a]	Genotype[b]			Allele[c]	*P*-value	
		A/A	A/G	G/G	MAF (%)	Genotype	Allele
Marker5	SCZ	305	175	23	22.0	0.662	0.576
	CON	271	154	15	20.9		

[a] SCZ=schizoprenia, CON=controls.
[b] M=major allele, m=minor allele.
[c] MAF=minor allele frequency.

controls=401). Marker-trait association showed an association Marker5 to SCZ ($P=0.0354$; genotype, $P=0.0122$; allele) (Table 1). However, after correction for the type I error rate by using program SNPSpD, corrected *P*-value became 0.204 for genotype and 0.0702 for allele, respectively (Table 2: effective number of independent loci, 5.7719, experiment-wide significance threshold required to keep type I error rate at 0.05, 0.008662).

To confirm Marker5 association, we performed a second-set analysis of Marker5 using an independent panel of samples (cases=503, controls=440). In this analysis, there was no association SCZ to Marker5 (Table 2).

We included a power calculation, and obtained more than 80% power to detect association when we set the genotype relative risk at 1.36 under a multiplicative model of inheritance.

4. Discussion

Through two-stage association analysis, htSNPs in TRAR4 were not found to be associated with SCZ in Japanese patients. Our results indicate the great importance of examining the possibility of false positives in genetic association analysis. False positives may be produced by population stratification. However, this might not be the case with our results, which instead may have derived from inflation of the type I error rate due to multiple testing, since the Japanese population is believed to be quite homogeneous.

We also included a haplotypic analysis of Marker5 and 6, which were relatively strong LD, using first-set samples (SAS/Genetics). Again, we could find no significant association ($P=0.0734$). This result also supports the possibility of a false positive for Marker5.

The strategy adopted in this study was a powerful one owing to the method of htSNP selection and two-stage association analysis. Moreover, by performing a mutation search with enough power to detect rare polymorphisms, we could avoid overlooking associations in accordance with the common disease–rare variant hypothesis (Pritchard, 2001).

In conclusion, we could not replicate the association of TRAR4 and SCZ using a Japanese population. Further replication analysis using different population samples will be required for conclusive results.

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References

Barrett, J.C., Fry, B., Maller, J., Daly, M.J., 2005. Haploview: analysis and visualization of LD and haplotype maps. Bioinformatics 21, 263–265.

Duan, J., Martinez, M., Sanders, A.R., Hou, C., Saitou, N., Kitano, T., Mowry, B.J., Crowe, R.R., Silverman, J.M., Levinson, D.F., Gejman, P.V., 2004. Polymorphisms in the trace amine receptor 4 (TRAR4) gene on chromosome 6q23.2 are associated with susceptibility to schizophrenia. Am. J. Hum. Genet. 75, 624–638.

Ikeda, M., Iwata, N., Suzuki, T., Kitajima, T., Yamanouchi, Y., Kinoshita, Y., Ozaki, N., 2005. No association of GSK3beta gene (GSK3B) with Japanese schizophrenia. Am. J. Med. Genet., B Neuropsychiatr. Genet. 134, 90–92.

Ke, X., Cardon, L.R., 2003. Efficient selective screening of haplotype tag SNPs. Bioinformatics 19, 287–288.

Nyholt, D.R., 2004. A simple correction for multiple testing for single-nucleotide polymorphisms in linkage disequilibrium with each other. Am. J. Hum. Genet. 74, 765–769.

Ohashi, J., Yamamoto, S., Tsuchiya, N., Hatta, Y., Komata, T., Matsushita, M., Tokunaga, K., 2001. Comparison of statistical power between 2*2 allele frequency and allele positivity tables in case–control studies of complex disease genes. Am. Hum. Genet. 65, 197–206.

Parker, E.M., Cubeddu, L.X., 1986. Effects of d-amphetamine and dopamine synthesis inhibitors on dopamine and acetylcholine neurotransmission in the striatum: I. Release in the absence of vesicular transmitter stores. J. Pharmacol. Exp. Ther. 237, 179–192.

Pritchard, J.K., 2001. Are rare variants responsible for susceptibility to complex diseases? Am. J. Hum. Genet. 69, 124–137.

Suzuki, T., Iwata, N., Kitamura, Y., Kitajima, T., Yamanouchi, Y., Ikeda, M., Nishiyama, T., Kamatani, N., Ozaki, N., 2003. Association of a haplotype in the serotonin 5-HT4 receptor gene (HTR4) with Japanese schizophrenia. Am. J. Med. Genet. 121B, 7–13.

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Association study of polymorphisms in the GluR7, KA1 and KA2 kainate receptor genes (*GRIK3*, *GRIK4*, *GRIK5*) with schizophrenia

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Abstract

On the basis of the glutamatergic dysfunction hypothesis of schizophrenia, we have been conducting a systematic study of the association of glutamate receptor genes with schizophrenia. Here we report association studies of schizophrenia with polymorphisms in three kainate receptor genes: *GRIK3*, *GRIK4* and *GRIK5*. We selected 16, 24 and 5 common single nucleotide polymorphisms (SNPs) distributed in the entire gene regions of *GRIK3* (>240 kb), *GRIK4* (>430 kb) and *GRIK5* (>90 kb), respectively. We tested associations of the polymorphisms with schizophrenia using 100 Japanese case–control pairs (the Kyushu set). We observed no significant "single marker" associations with the disease in any of the 45 SNPs tested except for one (rs3767092) in *GRIK3* showing a nominal level of significance. The significant association, however, disappeared after the application of the Bonferroni correction. We also observed significant haplotype associations in seven SNP pairs in *GRIK3* and in four SNP pairs in *GRIK4*. None, however, remained significant after Bonferroni correction. We also failed to replicate the nominally significant haplotype associations in a second sample set, the Aichi set (106 cases and 100 controls). We conclude that SNPs in the gene regions of *GRIK3*, *GRIK4* or *GRIK5* do not play a major role in schizophrenia pathogenesis in the Japanese population.

Keywords: Schizophrenia; Association study; Glutamate receptor; Kainate receptor gene; SNP; Linkage disequilibrium; Haplotype analysis

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1. Introduction

Reports of psychotic symptoms induced by phencyclidine (PCP) have stimulated interest in the possible role of a glutamatergic dysfunction in the pathogenesis of schizophrenia (Snyder, 1980; Javitt and Zukin, 1991). We have been conducting a systematic study of associations of the glutamate receptor (GluR) gene family with schizophrenia (Joo et al., 2001; Shibata et al., 2001, 2002; Tani et al., 2002; Makino et al., 2003; Fujii et al., 2003; Takaki et al., 2004). We have selected a set of common single nucleotide polymorphisms (SNPs) covering the entire genomic region of the target gene. By examining their allele frequencies and linkage disequilibria (LD), we tested individual associations and haplotype associations of the SNPs with the disorder. We earlier reported the absence of a significant association of schizophrenia with two genes encoding low-affinity kainate receptor subunits, GluR5 and GluR6 (Shibata et al., 2001, 2002). Here we report association studies of genes encoding the remaining three members of the kainate receptor subfamily, i.e., GluR7, KA1 and KA2, with schizophrenia.

The GluR7 receptor gene *GRIK3* is located on chromosome 1p34-33, where a significant linkage with schizophrenia has been reported (DeLisi et al., 2002). Significant changes of GluR7 expression in schizophrenia have been reported in multiple brain regions (Sokolov, 1998; Meador-Woodruff and Healy, 2000; Benes et al., 2001). The KA1 kainate receptor gene *GRIK4* is located on chromosome 11q22.3, where the fourth strongest linkage has been observed in a meta-analysis compiling 20 genome scans (Lewis et al., 2003). A significant decrease of KA1 mRNAs as well as of NMDAR1 and GluR1 mRNAs has been reported in the frontal cortex of neuroleptic-free patients with schizophrenia (Sokolov, 1998). The KA2 kainate receptor gene *GRIK5* is located on chromosome 19q13.2, where no linkage with schizophrenia has been previously reported. However, significant changes of KA2 expression in schizophrenia have been reported in multiple brain regions (Porter et al., 1997; Ibrahim et al., 2000; Meador-Woodruff and Healy, 2000). These lines of evidence suggest that *GRIK3*, *GRIK4* and *GRIK5* are strong candidates as susceptibility genes for schizophrenia.

In this report, we tested associations of schizophrenia with 16, 24 and 5 common SNPs selected from the entire regions of *GRIK3*, *GRIK4* and *GRIK5*, respectively. To enhance the detection power of haplotype association, the SNPs were placed depending on the magnitude of linkage disequilibrium (LD) in each subregion. That is, the weaker the observed LD, the more SNPs tested in the subregion. By testing haplotype associations in each subregion, we extensively examined associations of the entire gene regions with schizophrenia.

2. Methods

2.1. Subjects

Blood samples were obtained from unrelated Japanese individuals who provided written informed consent. We studied 100 schizophrenia patients recruited from hospitals in the Fukuoka and Oita areas (mean age=49.5; 44% female) and 100 healthy controls recruited from the Fukuoka area (mean age=51.2; 44% female) (the Kyushu set). To evaluate the significance of findings in the Kyushu set, we used another sample set, the Aichi set, which comprised 106 cases (mean age=39.4; 45.5% female) and 100 cases (mean age=34.4; 41.9% female) recruited from the Aichi area, about 600 km east of Fukuoka. All patients fulfilled the Diagnostic and Statistical Manual of Mental Disorders (DSM-IV) criteria for schizophrenia. This study was approved by the Ethics Committee of Kyushu University, Faculty of Medicine and by the Fujita Health University Ethics Committee. Genomic DNA was purified from leukocytes as previously described (Lahiri and Nurnberger, 1991).

2.2. SNP selection in the GRIK3, GRIK4 and GRIK5 regions

We retrieved all the primary information on SNPs from the public database, dbSNP (http://www.ncbi.nlm.nih.gov/SNP/) and a private database, Celera Discovery System (http://www.celeradiscoverysystem.com). We examined their allele frequencies in 16 healthy Japanese samples by the direct sequencing method as previously described (Shibata et al., 2002). When a tested SNP showed an insufficient

polymorphism (<10%), we selected another SNP close by the rare SNP and examined its allele frequency in the same way. Also we analyzed LD between the neighboring SNPs as described below. As we intended to cover the entire region with moderate-to-strong LD for haplotype association tests, we selected additional SNPs in the subregions showing very weak LD ($D' < 0.3$). For a direct test of functional variants, we also examined the frequency of all exonic SNPs available on the database and included them in the association analyses if they were common (>10%).

2.2.1. GRIK3

After the examination of 182 SNPs from the database, we selected the following 16 common SNPs distributed in the *GRIK3* region spanning over 240 kb: SNP1, rs551794; SNP 2, rs534131; SNP3, rs822856; SNP4, rs3767100; SNP5, rs3753771; SNP6, rs3767092; SNP7, rs3767086; SNP8, rs554445; SNP9, rs1160751; SNP10, rs1334804; SNP11, rs3767067; SNP12, rs550250; SNP13, rs565537; SNP14, rs3767048; SNP15, rs3767045; SNP16, rs2993076. All SNPs are located in noncoding regions except for one synonymous SNP, SNP12 in exon 3 (Fig. 1a).

2.2.2. GRIK4

Since we found a discrepancy at the 5′ ends in two GenBank entries, XM_166179 and NM_014619 for the *GRIK4* cDNA sequences, we selected both exons 1A and 1B as potential alternative transcription initiation sites. After the examination of 87 SNPs from the database, we selected the following 24 common SNPs distributed in the *GRIK4* region spanning over 430 kb: SNP1, rs2248404; SNP 2, rs1317176; SNP3, rs1317514; SNP4, rs1343789; SNP5, rs1893906; SNP6, rs2000870; SNP7, rs2000868; SNP8, rs1939664; SNP9, rs2852227; SNP10, rs3133226; SNP11, rs3133845; SNP12, rs2852230; SNP13, rs2846092; SNP14, rs2846103; SNP15, rs4359220; SNP16, rs4936552; SNP17, rs2004676; SNP18, rs3824978; SNP19, rs3802911; SNP20, rs2156637; SNP21, rs2156635; SNP22, rs2156634; SNP23, rs2298725; SNP24, rs611065. SNP19, SNP22 and

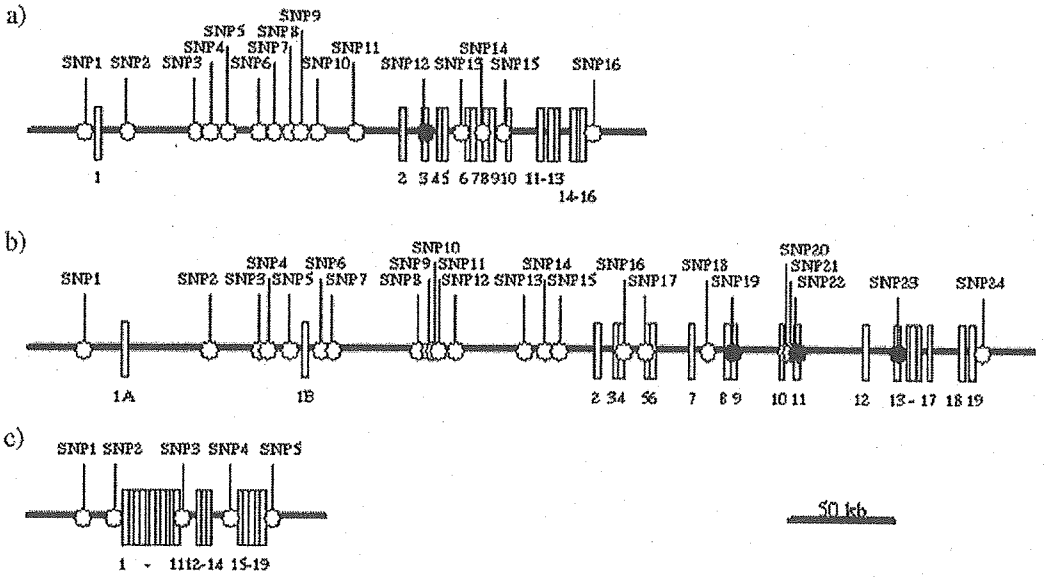

Fig. 1. Genomic organizations of *GRIK3*, *GRIK4* and *GRIK5*, and locations of the SNPs. (a) *GRIK3* spans over 233 kb and is composed of 16 exons shown as boxes with exon numbers. Circles indicate the 16 common SNPs we analyzed. (b) *GRIK4* spans over 410 kb and is composed of 19 exons shown as boxes with exon numbers. Circles indicate the 24 common SNPs we analyzed. (c) *GRIK5* spans over 67 kb and is composed of 19 exons shown as boxes with exon numbers. Circles indicate the 5 common SNPs we analyzed. Four exonic SNPs (one in *GRIK3* and three in *GRIK4*) are indicated by filled circles.

Table 1
PCR primers for genotyping of SNPs in *GRIK3, 4, 5*

Gene	SNP	Primer sequence (5′-3′)	Product size (bp)	Annealing temp (°C)	Cycle number	Typing method[a]
GRIK3	SNP1	Forward GGAAGGGAGCTGAAGGTAGG	211	63	35	RFLP
	rs551794	Reverse CCTGTGCACGTGTTTTGTTT				(*Bsp* HI)
	SNP2	Forward GGCTGTGTGAGGGCAGAC	354	60	30	RFLP
	rs534131	Reverse CCCGATTCTACTGGGACCTT				(*Pst* I)
	SNP3	Forward CCAGTGCCCAGAATCATATT	248	60	40	RFLP
	rs822856	Reverse GAAGGGCTGCAGTGAATGAC				(*Pvu* II)
	SNP4	Forward TCACCCTCGATGTAACCTTC	281	60	35	DS
	rs3767100	Reverse TTGCTTTCTAGCCGAGTCCA				
	SNP5	Forward AGGGAAGACACGGAACTCAC	330	60	35	DS
	rs3753771	Reverse ACACCTGGCATCAAGCATC				
	SNP6	Forward CCTCTTCCCCTCTCTTGCTT	291	60	40	RFLP
	rs3767092	Reverse ACAAAGGCTCGGCATCAG				(*Alu* I)
	SNP7	Forward CATGCACCTCACAATCAACC	186	60	35	RFLP
	rs3767086	Reverse AGCTGACCTGGGTTCCTACA				(*Hae* III)
	SNP8	Forward GAGCTCCTGCATCCTCAAAG	196	60	35	RFLP
	rs554445	Reverse CCAAGGAAAGCGAGTTCAAG				(*Pst* I)
	SNP9	Forward GATCTCCATGCTGCCTTTTC	386	55	35	DS
	rs1160751	Reverse TCAAGTGTCCCATCAATTCG				
	SNP10	Forward CCAGAACCATCCAACGAAGT	325	58	35	DS
	rs1334804	Reverse ATCTCAGGCACAAAGGGTGT				
	SNP11	Forward CCAGTTGGGATGGTGGTG	235	63	35	DS
	rs3767067	Reverse ATTATGCACAGCCAGGAGGA				
	SNP12	Forward GAGGGTTCCAGTTGCTGTTT	288	60	35	DS
	rs550250	Reverse GCGTAGTCGGGGTAGAGGTT				
	SNP13	Forward AGAGATGCTGCCCTTCACAC	308	58	30	RFLP
	rs565537	Reverse AACTGCCTTTCAACCAGGAG				(*Hha* I)
	SNP14	Forward ATAGCCTCCCTCTCCTCCAA	210	60	35	DS
	rs3767048	Reverse GCACCCATGCATACACTCAC				
	SNP15	Forward TCACTTCCTGACCCTGTCTTC	196	55	40	DS
	rs3767045	Reverse CTCTGGGCTTTGGCTGTT				
	SNP16	Forward CTCCCTTGAGGCGTGTTTT	182	58	40	DS
	rs2993076	Reverse AAGGACAGACCCCACATCCT				
GRIK4	SNP1	Forward CTGCAGTCTTTCTGCTGCAC	253	58	40	DS
	rs2248404	Reverse GGAAGCGCTAGATAGGTCGT				
	SNP2	Forward TCAAAGAGGGTTCCAGGATG	252	58	30	DS
	rs1317176	Reverse AGCAGACATAGCCCTGCTGT				
	SNP3	Forward AGCTGACACACTCCCTCACC	184	55	35	DS
	rs1317514	Reverse GCAAGCGTATAGAGGGGAAA				
	SNP4	Forward GCCTCGTTCCTTGACTGTGT	231	58	30	DS
	rs1343789	Reverse GGGCTCACCAAGGTCAGTAT				
	SNP5	Forward CAGAGACCCTCTGAGGTTGG	393	58	30	DS
	rs1893906	Reverse AATCACTTGAACCGGCAGAC				
	SNP6	Forward GGAACCAGACTGCTCGGATA	381	58	30	DS
	rs2000870	Reverse CCCTAGACAAGGTGGGGATT				
	SNP7	Forward CCTCTGAGACCTGCAGCAAT	247	63	30	DS
	rs2000868	Reverse CACCAGGCTGGAGCAGATAC				
	SNP8	Forward GGGAAAGGAAGGCTCAATTC	363	58	30	DS
	rs3133855	Reverse GCTATTGAAAGGCTGGGAGA				
	SNP9	Forward GGAGGCATCCATATTCAGGA	265	60	30	DS
	rs3133855	Reverse TGGATTCTCCCTCCTTCCTT				
	SNP10	Forward CAAGACAGCATTTTGGAGCA	283	58	30	DS
	rs3133226	Reverse TCCTCTTGGGAACTGTGAGG				

Table 1 (*continued*)

Gene	SNP	Primer sequence (5′-3′)	Product size (bp)	Annealing temp (°C)	Cycle number	Typing method[a]
	SNP11	Forward GCCCATGTCCTGAGTGAAAG	338	58	30	DS
	rs3133845	Reverse CCTCATGACTGCTTCCTTGC				
	SNP12	Forward GACTCAGGGACCCCAAGAA	242	58	30	DS
	rs2852230	Reverse ACCAGCCAGGAAAACATGAC				
	SNP13	Forward TGAGGGTGCTGCTCTAACAA	253	58	30	DS
	rs2846092	Reverse TCATTGGGATGGCCTCTATC				
	SNP14	Forward AGTTGCAACCTTGGCTCACT	352	63	40	DS
	rs2846103	Reverse CTTCCAGAGACCACCCTCAC				
	SNP15	Forward CTCCTGACCTCATGATCTGC	267	65	40	DS
	rs4359220	Reverse AGAGATCACGTGCTGCTGAC				
	SNP16	Forward CTGGGCTATCCCTGCCTAGA	345	63	30	DS
	rs4936552	Reverse ACAGACTTGGGTTTGCATCC				
	SNP17	Forward GATGGGGAATGATGCTGTTC	245	69	30	DS
	rs2004676	Reverse ATTCAGTGGGACGAGACAGG				
	SNP18	Forward ATCCACAAGGCTGTCCATTC	317	58	30	DS
	rs3824978	Reverse CCACTAGGCACATGGCTTTT				
	SNP19	Forward TTCTCCTACTCCAGGCCAAG	322	58	30	DS
	rs3802911	Reverse TTACCTGCCGAAAACCATTC				
	SNP20	Forward CCTGGGCTGAAATGATTGAT	222	58	30	DS
	rs2156637	Reverse TTTCCTGAACACCCGACAAT				
	SNP21	Forward AGCCTGGCTGAGTCCACTT	220	58	30	DS
	rs2156635	Reverse GGACAGGCAGACAAAGAAGG				
	SNP22	Forward CACCCCAAAGTAGCCCATTA	247	63	30	DS
	rs2156634	Reverse ATCTCTGCCAGCTCCTTGAG				
	SNP23	Forward GACAGGGAAGGGAAGAGGAG	258	65	30	DS
	rs2298725	Reverse ACCCACAGAAATGGGAACTG				
	SNP24	Forward TTGCCAGCCAGCTCTCTTAT	246	58	30	DS
	rs611065	Reverse GGAGGGCCACAAATGAGTCT				
GRIK5	SNP1	Forward GGAGGAGGGGAAGGATATGA	261	58	30	DS
	rs1056995	Reverse CCTTTCCCACTCAGAGACCA				
	SNP1.5	Forward CAGAGCCAGAAGTGGGAGAC	233	58	30	DS
	hCV1854167	Reverse CAGTGTCTCCTCCCCATCAC				
	SNP2	Forward TGTACTCCGAGCTTCCCAAG	330	60	30	DS
	rs4803523	Reverse GAACACTGGAGGAGGAGCTG				
	SNP4	Forward TGGTCTGAACAATGGGGAAT	229	58	30	DS
	rs8099939	Reverse CGAGTGGAGTTGCTGTCAGA				
	SNP5	Forward CTTCTGCAGCAAAGGAGGTT	186	60	30	DS
	rs4803520	Reverse GCCAAGGTGTCAAGAGAAGG				

[a] RFLP: restriction fragment length polymorphism; DS: direct sequencing.

SNP23 are synonymous SNPs located within exons 9, 11 and 13, respectively. The other 19 SNPs are located in noncoding regions (Fig. 1b).

2.2.3. *GRIK5*

After the examination of 46 SNPs from the database, we selected the following five common SNPs distributed in the *GRIK5* region spanning over 90 kb: SNP1, rs1056995; SNP 2, hCV1854167; SNP3, rs4803523; SNP4, rs8099939; SNP5, rs4803520. All SNPs are located in noncoding regions (Fig. 1c).

2.3. Genotyping of case–control pairs

All genotypes were determined either by direct sequencing or by polymerase chain reaction/restriction fragment length polymorphism (PCR-RFLP) as previously described (Shibata et al., 2001). Table 1 shows the nucleotide sequences of each primer, PCR conditions and genotyping methods for the 45 SNPs in the three gene regions. The raw data of direct sequencing were compiled on PolyPhred (Nickerson et al., 1997). The overall error rate was < 1%, estimated by compar-

Table 2
Genotype and allele frequencies of SNPs in *GRIK3*, *GRIK4*, and *GRIK5*

Polymorphism	Genotype count			p^a	Allele frequency (%)		p^b
a. *GRIK3*							
SNP1	AA	AT	TT		A	T	
Cases	3	21	76	0.3344	13.5	86.5	0.4420
Controls	0	21	79		10.5	89.5	
SNP2	AA	AG	GG		A	G	
Cases	37	44	19	0.8958	59	41	0.6828
Controls	39	45	16		61.5	38.5	
SNP3	AA	AC	CC		A	C	
Cases	78	18	4	0.1116	87	13	0.7606
Controls	77	23	0		88.5	11.5	
SNP4	CC	CT	TT		C	T	
Cases	1	13	86	0.4108	7.5	92.5	0.3004
Controls	1	20	79		11	89	
SNP5	CC	CT	TT		C	T	
Cases	1	15	84	0.4940	8.5	91.5	0.3227
Controls	1	22	77		12	88	
SNP6	AA	AG	GG		A	G	
Cases	12	45	43	0.0615	34.5	65.5	0.0319
Controls	19	53	28		45.5	54.5	
SNP7	GG	GT	TT		G	T	
Cases	77	22	1	0.4408	88	12	>0.9999
Controls	80	17	3		88.5	11.5	
SNP8	CC	CG	GG		C	G	
Cases	9	45	46	0.1204	31.5	68.5	0.0610
Controls	18	46	36		41	59	
SNP9	AA	AG	GG		A	G	
Cases	79	18	3	0.6886	88	12	0.8756
Controls	79	20	1		89	11	
SNP10	CC	CT	TT		C	T	
Cases	2	22	76	>0.9999	13	87	0.8800
Controls	1	22	77		12	88	
SNP11	CC	CT	TT		C	T	
Cases	84	15	1	0.8509	91.5	8.5	0.7304
Controls	81	18	1		90	10	
SNP12	CC	CT	TT		C	T	
Cases	1	16	83	>0.9999	9	91	>0.9999
Controls	1	17	82		9.5	90.5	
SNP13	AA	AG	GG		A	G	
Cases	4	19	77	0.4608	13.5	86.5	0.6505
Controls	1	21	78		11.5	88.5	
SNP14	AA	AG	GG		A	G	
Cases	66	30	4	0.3496	81	19	0.1331
Controls	76	22	2		87	13	
SNP15	GG	GT	TT		G	T	
Cases	86	14	0	0.6522	93	7	0.5809
Controls	84	14	2		91	9	
SNP16	CC	CT	TT		C	T	
Cases	39	49	12	0.1949	63.5	36.5	0.2439
Controls	51	37	12		69.5	30.5	
b. *GRIK4*							
SNP1	AA	AG	GG		A	G	
Cases	6	40	54	0.9002	26	74	0.7356
Controls	7	42	51		28	72	

Table 2 (*continued*)

Polymorphism	Genotype count			P^a	Allele frequency (%)		P^b
b. *GRIK4* (*continued*)							
SNP2	AA	AG	GG		A	G	
Cases	5	31	64	0.9081	20.5	79.5	0.8018
Controls	4	30	66		19	81	
SNP3	AA	AG	GG		A	G	
Cases	34	51	15	0.8535	59.5	40.5	0.6822
Controls	37	50	13		62	38	
SNP4	CC	CT	TT		C	T	
Cases	16	48	36	0.7434	40	60	0.8848
Controls	12	52	36		38	62	
SNP5	AA	AG	GG		A	G	
Cases	5	42	53	>0.9999	26	74	>0.9999
Controls	6	41	53		26.5	73.5	
SNP6	CC	CG	GG		C	G	
Cases	16	43	41	0.3552	37.5	62.5	0.2220
Controls	19	50	31		44	56	
SNP7	CC	CT	TT		C	T	
Cases	15	43	42	0.6967	36.5	63.5	0.5371
Controls	16	48	36		40	60	
SNP8	CC	CT	TT		C	T	
Cases	39	40	21	>0.9999	59	41	>0.9999
Controls	39	39	22		58.5	41.5	
SNP9	AA	AC	CC		A	C	
Cases	61	32	7	0.9750	77	23	>0.9999
Controls	61	31	8		76.5	23.5	
SNP10	CC	CT	TT		C	T	
Cases	31	49	20	0.8091	55.5	44.5	0.9200
Controls	32	45	23		54.5	45.5	
SNP11	AA	AG	GG		A	G	
Cases	27	52	21	0.6404	53	47	0.4839
Controls	25	48	27		49	51	
SNP12	CC	CG	GG		C	G	
Cases	27	52	21	0.1749	53	47	0.1334
Controls	23	44	33		45	55	
SNP13	AA	AG	GG		A	G	
Cases	16	49	35	0.3300	40.5	59.5	0.2559
Controls	9	51	40		34.5	65.5	
SNP14	CC	CT	TT		C	T	
Cases	4	35	61	0.4863	21.5	78.5	0.5321
Controls	5	27	68		18.5	81.5	
SNP15	AA	AG	GG		A	G	
Cases	65	33	2	0.2821	81.5	18.5	0.2782
Controls	72	28	0		86	14	
SNP16	AA	AT	TT		A	T	
Cases	31	49	20	0.6619	55.5	44.5	0.4228
Controls	26	50	24		51	49	
SNP17	CC	CT	TT		C	T	
Cases	25	48	27	0.9826	49	51	0.9203
Controls	25	46	29		48	52	
SNP18	AA	AG	GG		A	G	
Cases	12	38	50	0.2587	31	69	0.8300
Controls	8	49	43		32.5	67.5	
SNP19	CC	CT	TT		C	T	
Cases	26	58	16	0.1949	55	45	0.1094
Controls	17	59	24		46.5	53.5	

(continued on next page)

Table 2 (*continued*)

Polymorphism	Genotype count			P^a	Allele frequency (%)		P^b
b. *GRIK4* (*continued*)							
SNP20	AA	AG	GG		A	G	
Cases	23	50	27	0.7837	48	52	>0.9999
Controls	25	45	30		47.5	52.5	
SNP21	CC	CG	GG		C	G	
Cases	51	45	4	0.1180	73.5	26.5	0.6454
Controls	60	32	8		76	24	
SNP22	AA	AG	GG		A	G	
Cases	82	16	2	0.8848	90	10	0.7304
Controls	84	15	1		91.5	8.5	
SNP23	CC	CT	TT		C	T	
Cases	57	35	8	0.1991	74.5	25.5	0.1237
Controls	44	46	10		67	33	
SNP24	AA	AC	CC		A	C	
Cases	45	36	19	0.4286	63	37	0.6014
Controls	45	42	13		66	34	
c. *GRIK5*							
SNP1	CC	CT	TT		C	T	
Cases	79	19	2	0.7110	88.5	11.5	0.5491
Controls	75	22	3		86	14	
SNP2	CC	CG	GG		C	G	
Cases	10	37	53	0.7536	28.5	71.5	0.8233
Controls	7	40	53		27	73	
SNP3	CC	CT	TT		C	T	
Cases	61	31	8	0.3425	76.5	23.5	0.3943
Controls	64	33	3		80.5	19.5	
SNP4	GG	GT	TT		G	T	
Cases	39	41	20	0.9652	59.5	40.5	>0.9999
Controls	38	43	19		59.5	40.5	
SNP5	AA	AG	GG		A	G	
Cases	0	15	85	0.6796	7.5	92.5	>0.9999
Controls	1	12	87		7	93	

A significant P value is underlined.

[a] Fisher's exact probability tests, case vs. control (2 × 3, two-tailed).

[b] Fisher's exact probability tests, case vs. control (2 × 2, two-tailed).

ing the same samples genotyped by two different persons using direct sequencing and RFLP, respectively (data not shown).

2.4. Statistics

To control genotyping errors, Hardy–Weinberg equilibrium was checked in controls by the two-tailed χ^2 test ($df=1$). Statistical differences in genotype and allele frequencies between schizophrenic and control subjects were evaluated with Fisher's exact probability test (2 × 3 and 2 × 2, respectively). The normalized linkage disequilibrium statistic D' was calculated using haplotype frequencies estimated by the EH program, version 1.14 (Xie and Ott, 1993). Statistical analysis for the haplotype association was done by the two-tailed χ^2 test ($df=3$) according to Sham (1998). We excluded combinations of SNPs showing very weak LD ($D'<0.3$) from the haplotype analyses. The significance level for all statistical tests was 0.05. We applied the Bonferroni correction for all multiple tests.

3. Results

3.1. Single marker association analysis

We determined genotype and allele frequencies of the total 45 common SNPs in the three gene regions,

i.e., *GRIK3*, *GRIK4* and *GRIK5* (Table 2). The average allele frequencies of the SNPs are 0.18, 0.35 and 0.22, respectively. Given the equivalent frequency for the susceptible allele, the expected detection powers for *GRIK3*, *GRIK4* and *GRIK5* are 0.70 to 0.84, 0.84 to 0.94, and 0.75 to 0.89, respectively, under the multiplicative model with genotype relative risk = 1.8 to 2.0 (Ohashi and Tokunaga, 2001). Genotype frequencies of any of the 45 SNPs in controls did not show significant deviations from Hardy–Weinberg equilibrium (data not shown). Only the allele frequency of SNP6 in *GRIK3* showed a significant association with disease status ($P=0.0319$, Fisher's exact probability test). The nominal significance of the finding, however, did not survive the Bonferroni correction ($n=16$). We also failed to replicate the significance in the Aichi set ($P=0.686$, Fisher's exact probability test, data not shown).

3.2. Pairwise LD analysis

LD around common alleles can be measured with a modest sample size of 40–50 individuals to a precision within 10%–20% of the asymptotic limit (Reich et al., 2001). To evaluate the magnitude of LD, we calculated D' from the haplotype frequencies estimated by the EH program for all possible pairs of the tested SNPs within each gene region. Fig. 2 shows LD patterns within each gene region visualized by the GOLD program (Abecasis and Cookson, 2000). There was no essential difference in LD patterns in any of the genes between cases and controls. Since we selected more SNPs in low-LD subregions, we were able to localize several small subregions where LD drops abruptly. In the *GRIK3* region, we found one such LD gap between SNP14 and SNP15 (10.4 kb, $D'=0.0166$). In the *GRIK4* region, we found three gaps: SNP9–SNP10 (2.9 kb, $D'=0.2023$), SNP14–SNP15 (6.7 kb, $D'=0.091$), and SNP20–SNP21 (0.9 kb, $D'=0.1800$). No clear LD gaps were observed in the *GRIK5* region.

3.3. Haplotype association analysis

We intended to test haplotype associations with schizophrenia for all possible pairs of SNPs where

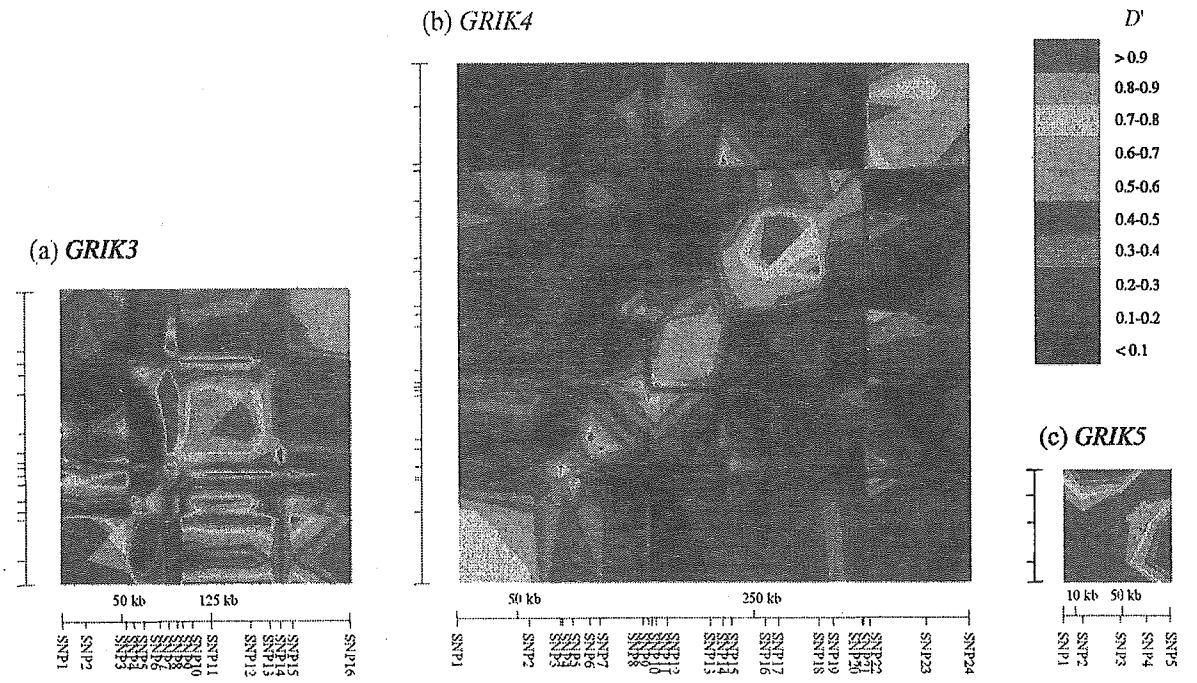

Fig. 2. LD patterns in the three gene regions of *GRIK3* (a), *GRIK4* (b) and *GRIK5* (c). The magnitude of LD (D') was visualized by the GOLD program (Abecasis and Cookson, 2000). The composite LD patterns in cases (upper diagonal) and in controls (lower diagonal) were drawn as squares in scale.

LD was maintained. To avoid the inflation of the number of statistical tests, we excluded 32, 141 and 1 SNP pairs from further analyses of *GRIK3*, *GRIK4* and *GRIK5*, respectively, because of very weak LD ($D'<0.3$) in both cases and controls. Out of a possible 120 pairs, we analyzed 88 pairs of SNPs showing moderate to strong LD in *GRIK3*. The following seven pairs showed significant haplotype associations with the disease: SNP1–SNP14 ($P=0.0391$, $df=3$, χ^2 test), SNP2–SNP6 ($P=0.0402$, $df=3$, χ^2 test), SNP3–SNP6 ($P=0.0166$, $df=3$, χ^2 test), SNP4–SNP6 ($P=0.0338$, $df=3$, χ^2 test), SNP5–SNP6 ($P=0.0196$, $df=3$, χ^2 test), SNP8–SNP14 ($P=0.0056$, $df=3$, χ^2 test), and SNP8–SNP15 ($P=0.0064$, $df=3$, χ^2 test) (Table 3a). However, none of the significant associations survived Bonferroni corrections ($n=88$). We also tested the most significant pair (SNP8–SNP14 of *GRIK3*) in the Aichi set, which failed to replicate the significance ($P=0.494$, $df=3$, χ^2 test; data not shown). We analyzed 90 pairs of SNPs out of 231 possible pairs in *GRIK4*. The following four pairs showed significant haplotype associations with the disease: SNP1–SNP19 ($P=0.0432$, $df=3$, χ^2 test), SNP2–SNP19 ($P=0.0242$, $df=3$, χ^2 test), SNP3–SNP4 ($P=0.0007$, $df=3$, χ^2 test), and SNP6–SNP19 ($P=0.0460$, $df=3$, χ^2 test) (Table 3b). Again none of the significant associations survived the Bonferroni correction ($n=90$). We also tested the most significant pair (SNP3–SNP4 of *GRIK4*) in the Aichi set, which failed to replicate the significance ($P=0.155$, $df=3$, χ^2 test; data not shown). We analyzed nine pairs of SNPs out of a possible 10 pairs in *GRIK5*. None of them showed significant haplotype associations with the disease (Table 3c).

4. Discussion

Our general approach to the testing of haplotype associations is based on haplotyping neighboring SNPs that are in moderate LD. We first genotyped both cases and controls for the SNPs evenly spaced. When insufficient LD was observed in the neighboring SNP pair ($D'<0.3$), additional SNPs were selected for genotyping within the LD gap. In other words, the candidate gene region is divided into multiple subregions where moderate LD is observed. Instead of haplotyping the entire gene region with many SNPs at once, we examined each subregion by haplotyping SNPs within the subregion so that we extensively tested the association of the entire gene with the disease (Shibata et al., 2002; Takaki et al., 2004). Through the SNP validation and LD analysis process, we selected 16, 24 and 5 common SNPs in the regions of *GRIK3*, *GRIK4* and *GRIK5* (Fig. 1). The average intervals of the SNPs were 16.1, 18.8 and 22.8 kb,

Table 3a
Pairwise haplotype association of SNPs in *GRIK3* with schizophrenia

	SNP1	SNP2	SNP3	SNP4	SNP5	SNP6	SNP7	SNP8	SNP9	SNP10	SNP11	SNP12	SNP13	SNP14	SNP15
SNP2	0.840														
SNP3	0.216	0.339													
SNP4	0.451	–	0.602												
SNP5	0.552	0.486	–	0.687											
SNP6	0.121	0.040	0.017	0.034	0.020										
SNP7	0.632	0.767	0.849	–	–	0.166									
SNP8	0.192	0.231	–	0.071	0.076	–	0.200								
SNP9	0.787	–	0.948	–	–	0.061	0.598	0.255							
SNP10	0.641	–	0.710	0.227	0.255	–	0.978	0.245	0.989						
SNP11	0.792	–	0.932	0.306	0.342	–	0.964	0.084	0.835	0.753					
SNP12	0.433	–	0.457	0.581	0.619	0.066	0.998	0.104	0.944	0.914	0.173				
SNP13	0.134	0.494	0.120	0.457	0.472	0.070	0.932	0.239	–	0.214	0.225	0.472			
SNP14	0.039	–	–	0.268	0.277	–	0.334	0.006	0.389	0.385	0.279	0.257	0.362		
SNP15	0.457	0.854	0.593	0.407	0.266	–	0.906	0.006	–	0.696	–	–	–	–	
SNP16	0.121	–	0.070	–	–	–	–	–	0.216	0.560	0.423	0.606	–	–	0.577

P values by the two-tailed χ^2 test for two-way haplotype association ($df=3$).
SNP pairs showing insufficient LD ($D'<0.3$) were not tested (denoted as "–").
Significant *P* values are underlined.

Table 3b
Pairwise haplotype association of SNPs in *GRIK4* with schizophrenia

	SNP1	SNP2	SNP3	SNP4	SNP5	SNP6	SNP7	SNP8	SNP9	SNP10	SNP11	SNP12	SNP13	SNP14	SNP15	SNP16	SNP17	SNP18	SNP19	SNP20	SNP21	SNP22	SNP23
SNP2	0.706																						
SNP3	–	0.854																					
SNP4	0.249	–	0.001																				
SNP5	–	–	0.259	0.052																			
SNP6	–	0.136	–	–	0.568																		
SNP7	0.593	0.410	–	–	–	0.063																	
SNP8	0.245	0.532	–	–	–	–	–																
SNP9	0.116	0.094	–	–	0.572	–	–	0.706															
SNP10	0.286	0.520	–	–	–	–	–	0.849	–														
SNP11	0.200	0.457	–	–	0.272	0.516	0.821	0.564	–	0.552													
SNP12	–	–	–	–	–	–	–	0.184	–	0.339	0.430												
SNP13	–	–	–	0.334	–	–	–	–	–	0.540	0.505	0.376											
SNP14	–	–	–	–	–	0.323	0.308	0.192	0.734	0.193	0.365	–	0.451										
SNP15	–	–	–	–	0.606	–	–	0.323	–	–	–	–	0.212	–									
SNP16	–	–	–	–	0.811	–	–	–	–	–	–	–	–	0.401	0.593								
SNP17	–	–	–	–	–	–	–	–	–	–	–	–	–	0.163	0.430	0.089							
SNP18	0.440	–	–	–	–	–	–	–	–	–	–	–	–	–	–	0.410	0.062						
SNP19	0.043	0.024	–	–	–	0.046	0.159	–	0.079	–	–	–	–	–	–	0.135	–	0.291					
SNP20	–	0.687	–	–	–	–	–	0.509	0.070	–	–	–	–	0.878	–	0.787	–	0.964	0.193				
SNP21	–	–	0.682	–	0.873	0.207	0.437	–	–	–	–	–	–	–	–	–	–	0.451	–	–			
SNP22	–	–	0.286	–	0.739	–	–	–	–	–	0.701	–	0.275	0.806	0.461	–	–	0.878	–	–	0.437		
SNP23	–	0.100	–	–	–	–	–	0.125	–	0.179	–	0.058	0.095	–	–	–	–	–	–	–	–	0.289	
SNP24	–	–	–	–	–	–	–	0.109	–	–	–	0.085	–	–	–	–	–	–	–	–	–	–	0.417

P value by the two-tailed χ^2 test for two-way haplotype association ($df=3$).
SNP-pairs showing insufficient LD ($D' < 0.3$) were not tested (denoted as "–").
Significant *P* values are underlined.

Table 3c
Pairwise haplotype association of SNPs in *GRIK5* with schizophrenia

	SNP1	SNP2	SNP3	SNP4
SNP2	0.896			
SNP3	0.739	0.619		
SNP4	0.479	0.407	0.479	
SNP5	0.724	–	0.577	0.999

P values by the two-tailed χ^2 test for two-way haplotype association ($df=3$).
SNP-pairs showing insufficient LD ($D'<0.3$) were not tested (denoted as "–").

respectively. Although we observed a nominal significance in the allele frequencies of SNP6 in *GRIK3* ($P=0.0319$, Fisher's exact probability test), there was no significant association observed in any of the other SNPs tested (Table 2). The significance of SNP6 in *GRIK3* was lost when we applied the Bonferroni correction ($n=16$). We also failed to replicate the significance in our second sample set, the Aichi set ($P=0.686$, Fisher's exact probability test). Therefore, we concluded that there is no common SNP showing a "single marker" association with schizophrenia in the 45 common SNPs of the three gene regions. We also examined an exonic SNP in *GRIK3*, rs6691840, for which a significant association with schizophrenia has been reported in the Italian population (Begni et al., 2002). Although we examined an equivalent sample size (100 cases and 100 controls) as in the study by Begni et al. (2002) (99 cases and 116 controls), the SNP was very rare (MAF=0.02) in our controls and completely monomorphic in our cases, indicating that there is no detectable association of the SNP with schizophrenia in the Japanese population. As we examined all of the exonic SNPs available on the database, there were no common exonic SNPs left untested for the association with the disease.

Seven pairs of SNPs in *GRIK3* and four pairs of SNPs in *GRIK4* showed significant haplotype associations with schizophrenia ($P=0.0007$, $df=3$, χ^2 test; see Tables 3a and b). However, none of them remained significant after Bonferroni correction. In addition, we failed to replicate the most significant pairs of SNPs, SNP8–SNP14 of *GRIK3* ($P=0.494$, $df=3$, χ^2 test) and SNP3–SNP4 of *GRIK4* ($P=0.155$, $df=3$, χ^2 test) in our second sample set, the Aichi set. We conclude that there is no significant haplotype association in any of the three regions, *GRIK3*, *GRIK4* and *GRIK5*.

We analyzed pairwise LD within each gene region (Fig. 2a–c). We observed no essential difference in LD patterns between cases and controls in any of the gene regions. Although we sampled SNPs more densely in weak LD subregions, we still observed four LD gaps. In the *GRIK3* region, one such subregion was found between SNP14 and SNP15 (10.4 kb, $D'=0.017$) where two exons, exon 8 and exon 9 as well as the entire intron 8, are located. Although we examined all other SNPs reported on the database within this subregion, none showed a high enough frequency for statistical testing (> 10%). Therefore, we could not analyze this subregion any further. In the *GRIK4* region, we found three gaps: SNP9–SNP10 (2.9 kb, $D'=0.202$), SNP14–SNP15 (6.7 kb, $D'=0.091$), and SNP20–SNP21 (0.9 kb, $D'=0.180$). The first two subregions are located within intron 1. The third subregion is located within intron 10. Although we tested all SNPs within the subregions reported on the database, none showed sufficient polymorphisms for statistical testing (> 10%). Therefore, we could not study these potential hot spots for recombination any further.

The remaining regions, which are the vast majority of the three genes, were successfully covered by LD as we intended. Assuming that no common SNPs were left untested in the LD gaps, those might have escaped from our haplotype analyses, there is no common variant of the three kainate receptor genes that is significantly associated with schizophrenia. We conclude that the three kainate receptor genes, *GRIK3*, *GRIK4* and *GRIK5*, do not play a major role in schizophrenia pathogenesis in Japanese.

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References

Abecasis, G.R., Cookson, W.O., 2000. GOLD—graphical overview of linkage disequilibrium. Bioinformatics 16, 182–183.

Begni, S., Popoli, M., Moraschi, S., Bignotti, S., Tura, G.B., Gennarelli, M., 2002. Association between the ionotropic glutamate receptor kainate 3 (*GRIK3*) ser310ala polymorphism and schizophrenia. Molecular Psychiatry 7, 416–418.

Benes, F.M., Todtenkopf, M.S., Kostoulakos, P., 2001. GluR5,6,7 subunit immunoreactivity on apical pyramidal cell dendrites in hippocampus of schizophrenics and manic depressives. Hippocampus 11, 482–491.

DeLisi, L.E., Mesen, A., Rodriguez, C., Bertheau, A., LaPrade, B., Llach, M., Riondet, S., Razi, K., Relja, M., Byerley, W., Sherrington, R., 2002. Genome-wide scan for linkage to schizophrenia in a Spanish-origin cohort from Costa Rica. American Journal of Medical Genetics 114, 497–508.

Fujii, Y., Shibata, H., Kikuta, R., Makino, C., Tani, A., Hirata, N., Shibata, A., Ninomiya, H., Tashiro, N., Fukumaki, Y., 2003. Positive associations of polymorphisms in the metabotropic glutamate receptor type 3 gene (*GRM3*) with schizophrenia. Psychiatric Genetics 13, 71–76.

Ibrahim, H.M., Hogg Jr., A.J., Healy, D.J., Haroutunian, V., Davis, K.L., Meador-Woodruff, J.H., 2000. Ionotropic glutamate receptor binding and subunit mRNA expression in thalamic nuclei in schizophrenia. American Journal of Psychiatry 157, 1811–1823.

Javitt, D.C., Zukin, S.R., 1991. Recent advances in the phencyclidine model of schizophrenia. American Journal of Psychiatry 148, 1301–1308.

Joo, A., Shibata, H., Ninomiya, H., Kawasaki, H., Tashiro, N., Fukumaki, Y., 2001. Structure and polymorphisms of the human metabotropic glutamate receptor type 2 gene (*GRM2*): analysis of association with schizophrenia. Molecular Psychiatry 6, 186–192.

Lahiri, D.K., Nurnberger, J.I. Jr., 1991. A rapid non-enzymatic method for the preparation of HMW DNA from blood for RFLP studies. Nucleic Acids Research 19, 5444.

Lewis, C.M., Levinson, D.F., Wise, L.H., DeLisi, L.E., Straub, R.E., Hovatta, I., Williams, N.M., Schwab, S.G., Pulver, A.E., Faraone, S.V., Brzustowicz, L.M., Kaufmann, C.A., Garver, D.L., Gurling, H.M., Lindholm, E., Coon, H., Moises, H.W., Byerley, W., Shaw, S.H., Mesen, A., Sherrington, R., O'Neill, F.A., Walsh, D., Kendler, K.S., Ekelund, J., Paunio, T., Lonnqvist, J., Peltonen, L., O'Donovan, M.C., Owen, M.J., Wildenauer, D.B., Maier, W., Nestadt, G., Blouin, J.L., Antonarakis, S.E., Mowry, B.J., Silverman, J.M., Crowe, R.R., Cloninger, C.R., Tsuang, M.T., Malaspina, D., Harkavy-Friedman, J.M., Svrakic, D.M., Bassett, A.S., Holcomb, J., Kalsi, G., McQuillin, A., Brynjolfson, J., Sigmundsson, T., Petursson, H., Jazin, E., Zoega, T., Helgason, T., 2003. Genome scan meta-analysis of schizophrenia and bipolar disorder. Part II. Schizophrenia. American Journal of Human Genetics 73, 34–48.

Makino, C., Fujii, Y., Kikuta, R., Hirata, N., Tani, A., Shibata, A., Ninomiya, H., Tashiro, N., Shibata, H., Fukumaki, Y., 2003. Positive association of the AMPA receptor subunit GluR4 gene (*GRIA4*) haplotype with schizophrenia: linkage disequilibrium mapping using SNPs evenly distributed across the gene region. American Journal of Medical Genetics 116B, 17–22.

Meador-Woodruff, J.H., Healy, D.J., 2000. Glutamate receptor expression in schizophrenic brain. Brain Research Reviews 31, 288–294.

Nickerson, D.A., Tobe, V.O., Taylor, S.L., 1997. Polyphred: automating the detection and genotyping of single nucleotide substitutions using fluorescence-based resequencing. Nucleic Acids Research 25, 2745–2751.

Ohashi, J., Tokunaga, K., 2001. The power of genome-wide association studies of complex disease genes: statistical limitations of indirect approaches using SNP markers. Journal of Human Genetics 46, 478–482.

Porter, R.H.P., Eastwood, S.L., Harrison, P.J., 1997. Distribution of kainate receptor subunit mRNA in human hippocampus, neocortex and cerebellum, and bilateral reduction of hippocampal GluR6 and KA2 transcripts in schizophrenia. Brain Research 751, 217–231.

Reich, D.E., Cargill, M., Bolk, S., Ireland, J., Sabeti, P.C., Richter, D.J., Lavery, T., Kouyoumjian, R., Farhadian, S.F., Ward, R., Lander, E.S., 2001. Linkage disequilibrium in the human genome. Nature 411, 199–204.

Sham, P., 1998. Statistics in Human Genetics. Oxford University Press, New York.

Shibata, H., Joo, A., Fujii, Y., Tani, A., Makino, C., Hirata, N., Kikuta, R., Ninomiya, H., Tashiro, N., Fukumaki, Y., 2001. Association study of polymorphisms in the coding region of the GluR5 kainate receptor gene (*GRIK1*) with schizophrenia. Psychiatric Genetics 11, 139–144.

Shibata, H., Shibata, A., Ninomiya, H., Tashiro, N., Fukumaki, Y., 2002. Association study of polymorphisms in the GluR6 kainate receptor gene (*GRIK2*) with schizophrenia. Psychiatry Research 113, 59–67.

Snyder, S.H., 1980. Phencyclidine. Nature 285, 355–356.

Sokolov, B.P., 1998. Expression of NMDAR1, GluR1, GluR7, and KA1 glutamate receptor mRNAs is decreased in frontal cortex of "neuroleptic-free" schizophrenics: evidence on reversible up-regulation by typical neuroleptics. Journal of Neurochemistry 71, 2454–2464.

Takaki, H., Kikuta, R., Shibata, H., Ninomiya, H., Tashiro, N., Fukumaki, Y., 2004. Positive associations of polymorphisms in the metabotropic glutamate receptor type 8 gene (*GRM8*) with schizophrenia. American Journal of Medical Genetics 128B, 6–14.

Tani, A., Kikuta, R., Itoh, K., Joo, A., Shibata, H., Ninomiya, H., Tashiro, N., Fukumaki, Y., 2002. Polymorphism analysis of upstream regions of the human *N*-methyl-D-aspartate receptor subunit NR1 gene (*GRIN1*): implications for schizophrenia. Schizophrenia Research 58, 83–86.

Xie, X., Ott, J., 1993. Testing linkage disequilibrium between a disease gene and marker loci. American Journal of Human Genetics 53, 1107.

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ORIGINAL ARTICLE

Haplotype association between $GABA_A$ receptor γ2 subunit gene (GABRG2) and methamphetamine use disorder

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ABSTRACT

Psychostimulant use disorder and schizophrenia have a substantial genetic basis. Evidence from human and animal studies on the involvement of the γ-aminobutyric acid (GABA) system in methamphetamine (METH) use disorder and schizophrenia is mounting. As we tested for the association of the human $GABA_A$ receptor gamma 2 subunit gene (GABRG2) with each diagnostic group, we used a case–control design with a set of 178 subjects with METH use disorder, 288 schizophrenics and 288 controls. First, we screened 96 controls and identified six SNPs in GABRG2, three of whom we newly reported. Next, we selected two SNPs, 315C>T and 1128+99C>A, as representatives of the linkage disequilibrium blocks for further case–control association analysis. Although no associations were found in either allelic or genotypic frequencies, we detected a haplotypic association in GABRG2 with METH use disorder, but not with schizophrenia. This finding partly replicates a recent case–control study of GABRG2 in METH use disorder, and thus indicates that GABRG2 may be one of the susceptibility genes of METH use disorder.

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INTRODUCTION

In recent years there has been a pronounced increase in use of psychostimulants involving methamphetamine (METH).[1] Lifetime prevalence of psychostimulant use in some developed countries is found in 1–3% of the adult population,[2] and psychostimulant use in any form may lead to abuse or dependence with physiological, psychological and behavioral component.[3] Findings from family and twin studies suggest that the genetic contribution is important for the development of psychostimulant use disorders. Heritability estimates from a population-based twin study for METH use disorder are substantial,[4,5] for example, 66% for psychostimulant abuse.[6]

The dopamine system is a prime candidate for genetic influence on drug abuse, particularly METH abuse, because it is thought to be involved in the reward and reinforcing mechanism in the meso-cortico-limbic system in the nucleus accumbens.[7] Moreover, the primary site of biological activity of METH is the dopamine transporter in this system.

Instead, a role for the γ-aminobutyric acid (GABA) system in drug abuse is also suggested in accumulating evidence. First, the irreversible GABA-transaminase inhibitor, γ-vinyl GABA, attenuates such increase of the dopamine release in the nucleus accumbens following acute administration of METH.[8] Second, QTL mapping for acute alcohol withdrawal severity suggests that a polymorphism in the $GABA_A$ receptor γ2 subunit gene in mice is genetically correlated with this phenotype.[9] A third line of evidence involves several case-control association studies, suggesting that the human $GABA_A$ receptor γ2 subunit gene (GABRG2) is marginally associated with METH use disorder,[10] and is also associated with alcoholism comorbid with antisocial personality disorder,[11] although there are conflicting results.[12,13] Therefore, it is possible that GABRG2 affects vulnerability to substance use disorder, including METH use disorder.

On the other hand, a number of post-mortem studies have reported an altered GABA neurotransmission in schizophrenia. These studies reported that release and uptake of GABA at synaptic terminals were reduced in schizophrenic cortex[14–16] and that the activity of glutamic acid decarboxylase (GAD), the synthesizing enzyme for GABA, GAD mRNA expression, and the density of GABAergic interneurons, were reduced in the prefrontal cortex (PFC) of schizophrenics.[17–20] Although there was reportedly no significant change in overall mRNA levels for $GABA_A$ receptor subunits,[17] expression of the alternately spliced short isoform of $GABA_A$ receptor γ2 subunit, γ2S, was markedly reduced in the PFC of schizophrenics.[21] The relative over-representation of the γ2L subunit, which possesses an additional phosphorylation site within the eight amino acids inserted, should result in a functionally less active form of the receptor,[22,23] and this defective GABAergic system may be involved in the development of schizophrenia. The evidence of linkage analysis from multiple genome scans of schizophrenia within 5q31–34 where GABRG2 locates also support the involvement of this gene in the development of schizophrenia.[24–27]

Here, we explored the possible contributions of GABRG2 in both METH use disorder and schizophrenia. We systemically searched all exons and the intronic branch sites of GABRG2 for polymorphisms, and examined haplotype-based case-control association analysis with both METH use disorder and schizophrenia.

RESULTS

Our screening of 96 controls in all exons and the flanking intronic splice sites of GABRG2 revealed six SNPs, which were designated 'Asn79Ser', '315C>T', '588T>C', '922+20G>A', '1129–1482A>C', and '1230C>T'. Minor allele frequencies and a schematic graph of these SNPs are presented in Table 1 and Figure 1, respectively. Of all identified SNPs, 315C>T, 588T>C (rs211037) and 922+20G>A have been reported elsewhere.

To evaluate the linkage disequilibrium (LD) in the 96 screened samples using several widely used measures (D', Δ_2 and *P*-value), we genotyped five SNPs in GABRG2 (two SNPs (315C>T, 588T>C) of identified SNPs, two SNPs (rs2268583, rs2284780) from the dbSNP database, and one SNP (1128+99C>A) reported as *Bam*HI RFLP previously[11]). These SNPs were selected because they showed sufficient heterozygosity (a frequency of minor allele>0.1) to detect a small effect of a susceptibility gene presumed to underlie complex disorders, and they were distributed almost evenly on the entire exonic regions of the gene (Figure 1).

Table 1 SNPs in GABRG2 and minor allele frequencies

SNP	*SNP position*	*Minor allele frequency*	*Reference*
107+740C>T	Intron 1	0.302	rs2268583
Asn79Ser	Exon 2	0.005	
315C>T	Exon 3	0.300	
588T>C	Exon 5	0.480	rs211037
922+20G>A	Intron 7	0.020	
923–466C>T	Intron 7	0.480	rs2284780
1128+99C>A	Intron 8	0.480	*Bam*HI C>A
1129–1482A>T	Intron 8	0.236	
1230C>T	Exon 9	0.005	

Estimation of LD between each pairwise SNP is presented in Table 2. These results show that the first three and the last two consecutive SNPs were in complete or nearly complete LD with each other. Therefore, we selected two SNPs (315C>T and 1128+99C>A) as representatives of these nearly complete LD regions for further case–control association analysis.

In addition to screened 96 samples, we genotyped 178 subjects with METH use disorder, 288 schizophrenics, and 288 controls in all. Two representative SNPs were in moderate LD with each other in METH use disorder ($D'=0.72$), schizophrenia ($D'=0.51$) and control subjects ($D'=0.61$). Genotypic and allelic frequencies of two SNPs in each population are summarized in Table 3. The genotypic distributions of each SNP did not significantly deviate from the Hardy–Weinberg equilibrium in either METH use disorder, schizophrenia or control subjects ($P=0.98$, 0.84 and 0.70 at 315C>T and $P=0.15$, 0.62 and 0.06 at 1128+99C>A, respectively). The distributions of each SNP did not differ significantly between each diagnostic group and controls in both allele and genotype frequencies (Table 3).

The distributions of haplotypic frequencies estimated using the expectation-maximization algorithm implemented in the Arlequin 2.0 significantly differed between METH use disorder and control subjects ($P=0.044$). In contrast, there was no significant difference in haplotypic distributions between schizophrenic and control subjects ($P=0.356$, Table 4). From examining at-risk haplotypes predisposed to METH use disorder, only two haplotypes, T–C and T–A (defined by 315C>T–1128+99C>A), were found to confer the significant susceptibility to this disorder. By applying the Bonferroni correction, this finding becomes nonsignificant for haplotype T–A (corrected $P=0.120$) and remains

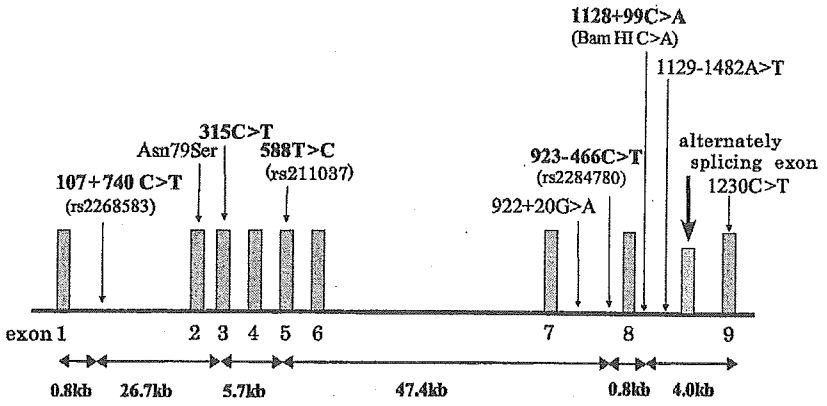

Figure 1 Schematic presentation of identified and reported GABRG2 SNPs. Solid box represents exons. The SNPs in bold type were used to evaluate LD structure.

Table 2 Pairwise linkage disequilibrium in controls

				D' Δ^2		
		rs2268583	*315C>T*	*588T>C*	*rs2284780*	*1128+99C>A*
P-value	rs2268583		1.000 0.976	0.926 0.457	0.376 0.058	0.376 0.058
	315C>T	$<1.0\times10^{-5}$		0.962 0.482	0.608 0.141	0.608 0.141
	588T>C	$<1.0\times10^{-5}$	$<1.0\times10^{-5}$		0.643 0.315	0.643 0.315
	rs2284780	0.002	0.0002	$<1.0\times10^{-5}$		1.000 1.000
	1128+99C>A	0.002	0.0002	$<1.0\times10^{-5}$	$<1.0\times10^{-5}$	

Table 3 Genotypic and allelic distributions of the GABRG2 SNPs in patients with METH use disorder and schizophrenia *vs* controls

SNP	*Sample*	*n*	*Genotype*			*Rarer allele*	*P-value*	
			CC	*CT*	*TT*	*T*	*Genotype*	*Allele*
315C>T	METH	178	87 (49%)	75 (42%)	16 (9%)	107 (30%)	0.374	0.174
	SCZ	288	151 (52%)	116 (40%)	21 (7%)	158 (27%)	0.818	0.594
	Control	288	157 (55%)	113 (39%)	18 (6%)	149 (26%)		
			CC	*CA*	*AA*	*A*	*Genotype*	*Allele*
1128+99C>A	METH	178	56 (31%)	79 (44%)	43 (24%)	165 (46%)	0.603	0.281
	SCZ	288	64 (22%)	139 (48%)	85 (30%)	309 (54%)	0.317	0.238
	Control	288	80 (28%)	128 (44%)	80 (28%)	288 (50%)		

Table 4 Haplotypic distributions of the GABRG2 gene in patients with METH use disorder and schizophrenia *vs* controls

Sample	*Haplotypes (315C>T–1128+99C>A)*				*P-value*
	C–C	C–A	T–C	T–A	
METH	0.275	0.425	0.262	0.039	0.044
SCZ	0.261	0.464	0.202	0.072	0.356
Control	0.314	0.428	0.186	0.072	

significant for haplotype T–C (corrected $P=0.028$). The presumed at-risk haplotype T–C has an estimated frequency of 18.6% among controls and 26.2% among METH use disorder subjects. The estimated odds ratio of haplotype T–C was 1.55 (95% CI (1.13–2.13)).

DISCUSSION

Our results provide supportive evidence for a haplotypic association in GABRG2 with METH use disorder, but not with schizophrenia. This association suggests that the susceptibility variant for METH use disorder may lie within the region in positive LD with the at-risk haplotype reconstructed in this study. The patterns of LD were shown to be two block like, the first block represented by 315C>T (covering rs2268583 at intron 1 to rs211037 at exon 5), and the second block represented by 1128+99C>A (covering rs2284780 at intron 7 to 1128+99C>A at intron 8). Since we found no association between each representative SNP and METH use disorder in either allelic or genotypic frequencies, the possibility arises that susceptibility variant can be located outside of these block-like regions. The second block includes the splicing regulatory elements surrounding the spliced exon, which bind to the polypyrimidine tract binding protein, the splicing regulator.[28–30] Actually, we screened this regulatory region thoroughly through direct sequencing of the 96 samples, however, could not find any variant in these elements. Other splicing regulatory elements that bind to another splicing regulator Nova-1 were located in intron 8, about 3.5 kb downstream of 1128+99C>A.[31,32] If the second block does not cover the latter splicing regulatory elements, these regions can be a susceptible candidate. Recently, a significant association was reported between rs4480617 at the 5′-UTR of GABRG2 and METH use disorder in females.[10] Therefore, this SNP or other variants in the promoter region also can be another candidate. Given that the sample size of 96 used to identify SNPs in this study provides more than 80% power to detect SNPs with about 1% minor allele frequency,[33] we are almost unlikely to overlook common nonsynonymous SNPs predisposed to METH use disorder.

As has been widely discussed, a spurious association can arise because of confounding such as population stratification and clinical heterogeneity, given the problems of reliability due to no use of structured interviews. However, our data are partly in agreement with a recent report[10] that found the significant association between GABRG2 and METH use disorder in females. This provides further corroboration that our haplotypic association with METH uses disorder is not spurious, although potential sources of bias such as ascertainment bias still remain possible. For example, subjects suffering from not only METH use disorder but also METH-induced psychosis are more likely to seek medical care and thus to be ascertained. Such 'spurious comorbidity'[34] of psychosis may account for the apparent association in this study. In the present study, we did not stratify the METH use disorder sample according to the comorbidity of METH-induced psychosis because the sample size was too small for reliable analysis. Although the precise prevalence of the comorbid METH-induced psychosis remains unknown, the data in the late 1940s and early 1950s in Japan indicating that about 10% of METH users had METH-induced psychosis[35] would suggest that comorbid METH-induced psychosis is over-represented in our clinically ascertained sample with METH use disorder.

As no association exists between GABRG2 and schizophrenia in our sample, association between GABRG2 and METH use disorder would not likely be attributable to spurious comorbid METH induced-psychosis, which may share the pathophysiology of susceptibility with schizophrenia, the so-called sensitization phenomena.[35] On the contrary, the comorbid polysubstance-related disorder over-represented in our sample with METH use disorder can account for the apparent association in this study. Indeed, previous findings suggesting nonspecific substance dependence vulnerability[5] supported the existence of such a 'misattributed' association in our study. In addition to concurrent comorbidity, we also cannot deny the possibility of spurious comorbid bias caused by the past comorbid diseases because of not examining the past history of any mental diseases systematically. METH use subjects in our study included a large number of patients who experienced first psychotic symptoms after METH use for a relatively short duration and participants in the special program designed for drug use disorder, in which they could not participate if they suffered from other psychiatric problems. The low levels of comorbidity in METH use subjects may reflect such biased ascertainment.

There is indeed a neuroscientific framework to link GABRG2 and METH use disorder. First, several lines of investigation[7] implicate the mesolimbic dopamine system in psychostimulant-induced motor activity. Furthermore, it was shown in a pharmacological study[36] that a GABAergic system in PFC modulated the motor response to psychostimulants by inhibiting PFC pyramidal neurons. Second, a tentative association was found for a GABRG2 SNP and the frontally located event-related potential (ERP) complex N100/P200 after auditory stimuli.[37] Thus, the prefrontal activation difference may reflect the differential GABRG2 activities derived from variants of the gene. Accordingly, GABRG2 activities in PFC could affect the modulation of mesolimbic reward circuitries, which might be associated with vulnerability of METH use disorder.

Overall our results indicate that GABRG2 may play a role in the risk of METH use disorder development in this population. Analysis of the promoter region or the splicing regulatory elements in intron 8 in a future study would be a logical next step in searching for a susceptible variant of GABRG2 in METH use disorder. However, it remains uncertain whether the associated phenotype may reflect the vulnerability of METH-specific abuse or nonspecific substance abuse.

METHODS

Subjects

All patients in this study were unrelated and recruited from three medical institutes participating the Japanese Genetics Initiative for Drug Abuse (JGAIDA).[38] They were diagnosed according to DSM-IV criteria by the consensus of at least two experienced psychiatrists on the basis of unstructured interviews and review of the medical records prior to genotyping.

The number of the patients with METH uses disorder, comprised of 164 METH-dependent subjects, and 14 METH abuse subjects, and schizophrenia were 178 (144 males and 34 females) and 288 (140 males and 148 females), respectively. The ages of each patient group were 18–69 years old (mean$\pm$SD; 36.7$\pm$12.0) and 15–75 (39.6$\pm$14.0), respectively. No patient with schizophrenia had severe physical complications or other Axis-I disorders according to DSM-IV when enrolled in this study, because seven schizophrenic subjects with METH use disorder were excluded based on the criteria that restricted a comorbid diagnosis of any psychotic disorder other than METH-induced psychosis. Among the subjects with METH use disorder, 150 (124 males and 25 females) have a comorbid diagnosis of METH-induced psychosis, three of anorexia nervosa, one of obsessive-compulsive disorder, and one of major depressive disorder. Additionally, 119 subjects with METH use disorder have abuse or dependence on drugs other than METH. The past history of any mental illness was not examined. The ages of METH-induced psychotic subgroup were 19–69 years old (37.7$\pm$12.3). No patient with METH use disorder had any severe physical complications when enrolled in this study. The 288 unrelated healthy volunteers (152 males and 136 females), aged 19–65 years (33.6$\pm$13.0), were comprised of hospital staff members and medical students at Fujita Health University. All healthy controls were also psychiatrically screened based on unstructured interviews. After complete description of the study to each subject, written informed consent was obtained. This study was approved by the ethics committee of each JGAIDA institute.

SNP Identification

Genomic DNA was isolated from whole blood using PUREGNER (Gentra system, Minneapolis, MN 55447, USA). For denaturing high-performance liquid chromatography (DHPLC) analysis, we designed specific primer sets amplifying all GABRG2 exons and the flanking intronic splice sites, based on GenBank sequence (NM000816 and NT023133) (primer sequences are available on request).

Polymerase chain reaction (PCR) was performed in a 10-μl volume containing 10 ng sample DNA, 0.4 M of each primer, 200 μM each dNTP, 1 × PCR Gold Buffer, 1.5 mM $MgCl_2$ and 0.25 U of Amplitaq Gold™ (Applied Biosystems Japan Ltd, Tokyo, Japan), using GeneAmp™ PCR system 9700 (Applied Biosystems Japan Ltd). PCR cycling conditions consisted of an initial denaturation step at 95°C for 9 min, followed by 45 cycles of 95°C for 15 s, 60°C for 20 s, 72°C for 30 s, and ending with a final extension step at 72°C for 7 min.

To screen for nucleotide variants, the obtained PCR products from all screened samples were analyzed by DHPLC with the WAVE™ system (Transgenomics Japan Ltd, Tokyo, Japan). The PCR products showing variant chromatograms were amplified again and then sequenced with an ABI PRISM™ 3100 Genetic Analyzer (Applied Biosystems Japan Ltd). Furthermore, to screen for any kinds of nucleotide variants in the splicing regulatory elements surrounding the spliced exon, we performed direct sequencing of the 96 controls. The conditions for DHPLC analysis and direct sequencing were reported previously.[39]

SNP Genotyping

To confirm the sequencing result and to genotype the variants in additional samples, the DHPLC analysis using the primer extension methods were developed for genotyping 588T>C by modifying the method of Hoogendoorn *et al*,[40] as reported previously.[39] All the remaining SNPs examined were genotyped using PCR-restriction fragment length polymorphism (PCR-RFLP) methods. Of four RFLP sites selected, the *Bam*HI restriction site in the eighth exon was genotyped as described by Loh *et al*,[12] while for the rest of the three SNPs, PCR-RFLP methods were developed (detailed information on experimental procedures is available upon request).

Statistical Analysis

Tests for Hardy–Weinberg equilibrium, the calculation of LD measures such as D′, Δ_2 and *P*-value and the estimation of haplotypic frequencies were carried out using Arlequin software 2.0.[41] The haplotypic frequencies between each patient group and controls were also compared using Arlequin software 2.0. The genotypic and allelic frequencies among each patient group and control group were compared with an exact test, using SPSS (version 10). A two-tailed level of 5% was chosen for the type I error rate. We have not corrected for multiple testing so as to avoid false negative findings.

Following Ohashi and Tokunaga,[40] we estimated the power of association analysis for our sample size of 178 subjects with METH use disorder, 288 schizophrenics and 288 controls under multiplicative model of inheritance, assuming a population susceptibility allele frequency of 0.30 at 315C>T and 0.48 at 1128+99C>A, the value in our screened samples. Setting the type I error rate at 5% and Genotype relative risk at more than 1.4 and 1.5, we obtained more than 80% power for direct association analysis of METH use disorder and schizophrenia, respectively.

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DUALITY OF INTEREST

None declared.

ABBREVIATIONS

METH	*methamphetamine*
GABA	*γ-aminobutyric acid*
GABRG2	*The human $GABA_A$ receptor gamma 2 subunit gene*
GAD	*glutamic acid decarboxylase*
PFC	*prefrontal cortex*
LD	*linkage disequilibrium*
DHPLC	*denaturing high-performance liquid chromatography*
PCR-RFLP	*polymerase chain reaction-restriction fragment length polymorphism*

REFERENCES

1 Farrell M, Marsden J, Ali R, Ling W. Methamphetamine: drug use and psychoses becomes a major public health issue in the Asia Pacific region. *Addiction* 2002; **97**: 771–772.
2 UNDCP. *United National International Drug Control Programme (UNDCP): World Drug Report*. Oxford University Press: New York, 1997.
3 Sato M, Chen CC, Akiyama K, Otsuki S. Acute exacerbation of paranoid psychotic state after long-term abstinence in patients with previous methamphetamine psychosis. *Biol Psychiatry* 1983; **18**: 429–440.
4 Tsuang MT, Lyons MJ, Eisen SA, Goldberg J, True W, Lin N *et al*. Genetic influences on DSM-III-R drug abuse and dependence: a study of 3372 twin pairs. *Am J Med Genet* 1996; **67**: 473–477.
5 Tsuang MT, Lyons MJ, Meyer JM, Doyle T, Eisen SA, Goldberg J *et al*. Co-occurrence of abuse of different drugs in men: the role of drug-specific and shared vulnerabilities. *Arch Gen Psychiatry* 1998; **55**: 967–972.
6 Kendler KS, Karkowski LM, Neale MC, Prescott CA. Illicit psychoactive substance use, heavy use, abuse, and dependence in a US population-based sample of male twins. *Arch Gen Psychiatry* 2000; **57**: 261–269.
7 Spanagel R, Weiss F. The dopamine hypothesis of reward: past and current status. *Trends Neurosci* 1999; **22**: 521–527.
8 Gerasimov MR, Dewey SL. Gamma-vinyl gamma-aminobutyric acid attenuates the synergistic elevations of nucleus accumbens dopamine produced by a cocaine/heroin (speedball) challenge. *Eur J Pharmacol* 1999; **380**: 1–4.
9 Buck KJ, Finn DA. Genetic factors in addiction: QTL mapping and candidate gene studies implicate GABAergic genes in alcohol and barbiturate withdrawal in mice. *Addiction* 2001; **96**: 139–149.
10 Lin SK, Chen CK, Ball D, Liu HC, Loh EW. Gender-specific contribution of the GABA(A) subunit genes on 5q33 in methamphetamine use disorder. *Pharmacogenomics J* 2003; **3**: 349–355.
11 Loh EW, Higuchi S, Matsushita S, Murray R, Chen CK, Ball D. Association analysis of the GABA(A) receptor subunit genes cluster on 5q33–34 and alcohol dependence in a Japanese population. *Mol Psychiatry* 2000; **5**: 301–307.
12 Loh EW, Smith II, Murray R, McLaughlin M, McNulty S, Ball D. Association between variants at the $GABA_A$beta2, $GABA_A$alpha6 and $GABA_A$gamma2 gene cluster and alcohol dependence in a Scottish population. *Mol Psychiatry* 2000; **5**: 452.
13 Sander T, Ball D, Murray R, Patel J, Samochowiec J, Winterer G *et al*. Association analysis of sequence variants of GABA(A) alpha6, beta2, and gamma2 gene cluster and alcohol dependence. *Alcohol Clin Exp Res* 1999; **23**: 427–431.
14 Simpson MD, Slater P, Deakin JF, Royston MC, Skan WJ. Reduced GABA uptake sites in the temporal lobe in schizophrenia. *Neurosci Lett* 1989; **107**: 211–215.
15 Reynolds GP, Czudek C, Andrews HB. Deficit and hemispheric asymmetry of GABA uptake sites in the hippocampus in schizophrenia. *Biol Psychiatry* 1990; **27**: 1038–1044.
16 Sherman AD, Davidson AT, Baruah S, Hegwood TS, Waziri R. Evidence of glutamatergic deficiency in schizophrenia. *Neurosci Lett* 1991; **121**: 77–80.
17 Akbarian S, Huntsman MM, Kim JJ, Tafazzoli A, Potkin SG, Bunney Jr WE *et al*. $GABA_A$ receptor subunit gene expression in human prefrontal cortex: comparison of schizophrenics and controls. *Cereb Cortex* 1995; **5**: 550–560.
18 Volk DW, Austin MC, Pierri JN, Sampson AR, Lewis DA. Decreased glutamic acid decarboxylase67 messenger RNA expression in a subset of prefrontal cortical gamma-aminobutyric acid neurons in subjects with schizophrenia. *Arch Gen Psychiatry* 2000; **57**: 237–245.
19 Gluck MR, Thomas RG, Davis KL, Haroutunian V. Implications for altered glutamate and GABA metabolism in the dorsolateral prefrontal cortex of aged schizophrenic patients. *Am J Psychiatry* 2002; **159**: 1165–1173.
20 Guidotti A, Auta J, Davis JM, Di-Giorgi-Gerevini V, Dwivedi Y, Grayson DR *et al*. Decrease in reelin and glutamic acid decarboxylase67 (GAD67) expression in schizophrenia and bipolar disorder: a postmortem brain study. *Arch Gen Psychiatry* 2000; **57**: 1061–1069.
21 Huntsman MM, Tran BV, Potkin SG, Bunney Jr WE, Jones EG. Altered ratios of alternatively spliced long and short gamma2 subunit mRNAs of the gamma-amino butyrate type A receptor in prefrontal cortex of schizophrenics. *Proc Natl Acad Sci USA* 1998; **95**: 15066–15071.
22 Kofuji P, Wang JB, Moss SJ, Huganir RL, Burt DR. Generation of two forms of the gamma-aminobutyric acidA receptor gamma 2-subunit in mice by alternative splicing. *J Neurochem* 1991; **56**: 713–715.
23 Whiting P, McKernan RM, Iversen LL. Another mechanism for creating diversity in gamma-aminobutyrate type A receptors: RNA splicing directs expression of two forms of gamma 2 phosphorylation site. *Proc Natl Acad Sci USA* 1990; **87**: 9966–9970.
24 Straub RE, MacLean CJ, O'Neill FA, Walsh D, Kendler KS. Support for a possible schizophrenia vulnerability locus in region 5q22–31 in Irish families. *Mol Psychiatry* 1997; **2**: 148–155.
25 Kendler KS, Myers JM, O'Neill FA, Martin R, Murphy B, MacLean CJ *et al*. Clinical features of schizophrenia and linkage to chromosomes 5q, 6p, 8p, and 10p in the Irish Study of High-Density Schizophrenia Families. *Am J Psychiatry* 2000; **157**: 402–408.
26 Gurling HM, Kalsi G, Brynjolfson J, Sigmundsson T, Sherrington R, Mankoo BS *et al*. Genomewide genetic linkage analysis confirms the presence of susceptibility loci for schizophrenia, on chromosomes 1q32.2, 5q33.2, and 8p21–22 and provides support for linkage to schizophrenia, on chromosomes 11q23.3–24 and 20q12.1–11.23. *Am J Hum Genet* 2001; **68**: 661–673.
27 Lewis CM, Levinson DF, Wise LH, DeLisi LE, Straub RE, Hovatta I *et al*. Genome scan meta-analysis of schizophrenia and bipolar disorder, part II: Schizophrenia. *Am J Hum Genet* 2003; **73**: 34–48.
28 Zhang L, Ashiya M, Sherman TG, Grabowski PJ. Essential nucleotides direct neuron-specific splicing of gamma 2 pre-mRNA. *RNA* 1996; **2**: 682–698.
29 Zhang L, Liu W, Grabowski PJ. Coordinate repression of a trio of neuron-specific splicing events by the splicing regulator PTB. *RNA* 1999; **5**: 117–130.
30 Ashiya M, Grabowski PJ. A neuron-specific splicing switch mediated by an array of pre-mRNA repressor sites: evidence of a regulatory role for the polypyrimidine tract binding protein and a brain-specific PTB counterpart. *RNA* 1997; **3**: 996–1015.
31 Jensen KB, Dredge BK, Stefani G, Zhong R, Buckanovich RJ, Okano HJ *et al*. Nova-1 regulates neuron-specific alternative splicing and is essential for neuronal viability. *Neuron* 2000; **25**: 359–371.
32 Dredge BK, Darnell RB. Nova regulates GABA(A) receptor gamma2 alternative splicing via a distal downstream UCAU-rich intronic splicing enhancer. *Mol Cell Biol* 2003; **23**: 4687–4700.
33 Collins JS, Schwartz CE. Detecting polymorphisms and mutations in candidate genes. *Am J Hum Genet* 2002; **71**: 1251–1252.
34 Smoller JW, Lunetta KL, Robins J. Implications of comorbidity and ascertainment bias for identifying disease genes. *Am J Med Genet* 2000; **96**: 817–822.