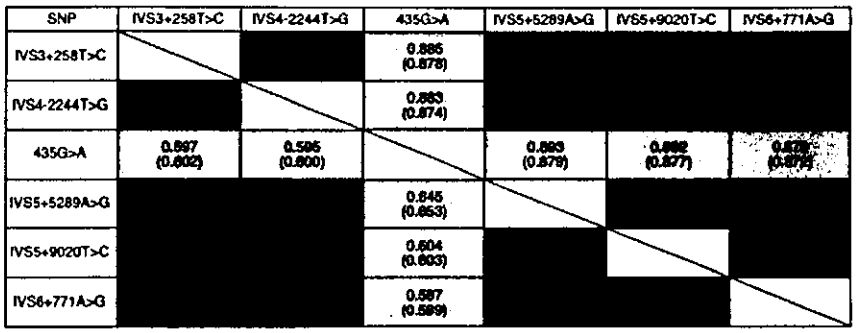

Figure 2. Pairwise linkage disequilibrium estimations between single nucleotide polymorphisms (SNPs) in *FZD3*. Upper-right diagonal shows standardized D' values, and the lower left shows r^2 (squared correlation coefficient) in control and schizophrenia (in parentheses) groups.

using Fisher's Exact Test. Comparisons of gene expression were performed using Mann–Whitney U tests.

Results

None of the three SNPs (435G>A, IVS5+5289A>G, and IVS5+9020*T*>C) that displayed preferential transmissions to patients in Chinese families (Yang et al 2003a) exhibited significant transmission disequilibrium in our family samples, either by pedigree disequilibrium test (for all families) or extended transmission disequilibrium (for 168 independent and complete trios; Table 1), nor did the three additional SNPs (IVS3+258*T*>C, IVS4–2244*T*>G, and IVS6+771A>G) newly analyzed in this study (Table 1). Moreover, haplotype transmission analysis found no SNP-based haplotypes that were preferentially transmitted to schizophrenia patients (Table 1). Our family sample and those of Yang et al (2003a) had power of .71 and .76, respectively, to detect significant associations, based on models assuming that the genotypic relative risk is 1.5 with an additive inheritance mode. Within the same assumption, our case–control panel had a power of .98.

Genotypic distributions of six SNPs in all our samples displayed Hardy–Weinberg equilibrium. In case–control analysis, no significant differences existed between schizophrenia patients and control subjects in allelic or genotypic distributions of the six SNPs or the three SNP-based haplotypes (Table 1). No evidence indicated population stratification in our case–control samples [$Pr(K = 1) > .99$].

Pairwise LD was calculated between SNP markers in case–control samples (Figure 2). All D' measures were >.8 and r^2 measures were >.5, suggesting that the six SNPs display strong LD to each other and are on the same LD block (Abecasis et al 2001; Nakajima et al 2002). These results are concordant with the HapMap data (http://www.broad.mit.edu/personal/jcbarret/haploview/index.php).

Expression of *FZD3* in the DLPFC, an important brain region in terms of schizophrenia pathology (Cannon et al 2002), did not differ significantly between schizophrenic and control brains (Figure 3), although a possible modulatory effect of medication cannot be excluded.

Discussion

Evidence for potential disturbances of Wnt signaling pathways in schizophrenia has accumulated (Cotter et al 1998; Emamian et al 2004; Lijam et al 1997; Miyaoka et al 1999); however, our results do not support prior association results for Wnt molecular component FZD3, in either family- or population-based designs. Polymorphisms in the promoter region could alter gene expression, but we observed no differences in levels of *FZD3* expression between schizophrenic and control brains. One possible reason for this discrepancy may be differences in extent of LD around the *FZD3* locus between Chinese and Japanese populations. The association signals detected by Yang et al (2003a) might be due to nearby genes that are in strong LD with *FZD3* but not present in our Japanese sample. Yang et al (2003b) also reported associations between Neuregulin 1 gene (*NRG1*) on 8p21 and schizophrenia; however, risk haplotypes of *NRG1* and *FZD3* are unlikely to exist on the same LD block, because the two genes are about 3 Mb apart, too far to preserve substantial LD. Chinese and Japanese populations are genetically close (Saitou 1995), and this does not favor differing LD structures as a cause of discrepancy. Another possibility may be heterogeneous etiologies in schizophrenia. Meiotic drive can cause preferential transmission of particular genetic variants (Zollner et al 2004); therefore it would be important to exclude this phenomenon in their study by examining transmissions to healthy offspring.

Katsu et al (2003) reported associations between schizophrenia and IVS3+258*T*>C, but not 435G>A, in *FZD3* in a Japanese sample. Those results may be inconsistent because the two polymorphisms are in close LD to each other in Japanese populations. Excluding possible statistical fluctuations due to small sample sizes or population stratification in their study would thus be important.

In conclusion, we found no major genetic contribution of *FZD3* to risk for schizophrenia.

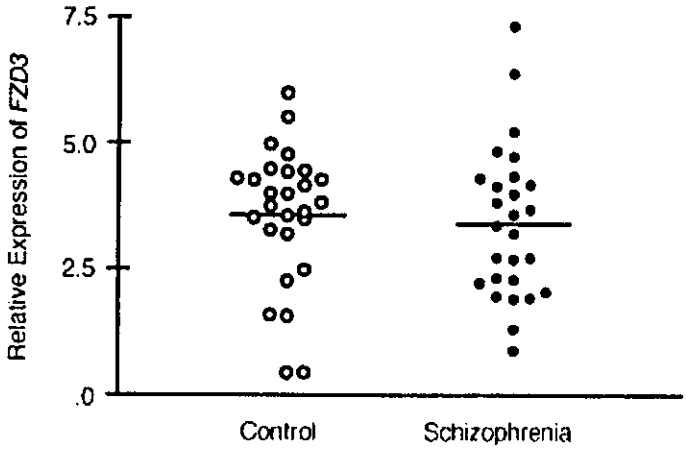

Figure 3. *FZD3* mRNA levels in postmortem brains (Brodmann's area 46) from schizophrenic and control subjects. Expression level of *FZD3* is normalized against that of β2-microgbulin. Horizontal bars indicate means. No significant differences in expression were found between schizophrenic and control subjects ($p = .416$).

This work was supported by RIKEN Brain Science Institute Funds, Research on Brain Science Funds from the Ministry of Health Labor and Welfare, and CREST funds from the Japan Science and Technology Agency, Japan. Postmortem brains were donated by the Stanley Medical Research Institute's Brain Collection, courtesy of Drs. Knable, Torrey, Webster, Weis, and Yolken.

Abecasis GR, Noguchi E, Heinzmann A, Traherne JA, Bhattacharyya S, Leaves NI, et al (2001): Nucleotide diversity and haplotype structure of the human angiotensin gene in two populations. *Am J Hum Genet* 70:108–123.

Blouin JL, Dombroski BA, Nath SK, Lasseter VK, Wolyniec PS, Nestadt G, et al (1998): Schizophrenia susceptibility loci on chromosomes 13q32 and 8p21. *Nat Genet* 20:70–73.

Cannon TD, Thompson PM, van Erp TG, Toga AW, Poutanen VP, Huttunen M, et al (2002): Cortex mapping reveals regionally specific patterns of genetic and disease-specific gray-matter deficits in twins discordant for schizophrenia. *Proc Natl Acad Sci U S A* 99:3228–3233.

Clayton D (1999): A generalization of the transmission/disequilibrium test for uncertain-haplotype transmission. *Am J Hum Genet* 65:1170–1177.

Cotter D, Kerwin R, al-Sarraji S, Brion JP, Chadwich A, Lovestone S, et al (1998): Abnormalities of Wnt signalling in schizophrenia–evidence for neurodevelopmental abnormality. *Neuroreport* 9:1379–1383.

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

Emamian ES, Hall D, Birnbaum MJ, Karayiorgou M, Gogos JA (2004): Convergent evidence for impaired AKT1-GSK3beta signaling in schizophrenia. *Nat Genet* 36:131–137.

Gurling HM, Kalsi G, Brynjolfson J, Sigmundsson T, Sherrington R, Mankoo BS, et al (2001): 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* 68:661–673.

Katsu T, Ujike H, Nakano T, Tanaka Y, Nomura A, Nakata K, et al (2003): The human frizzled-3 (FZD3) gene on chromosome 8p21, a receptor gene for Wnt ligands, is associated with the susceptibility to schizophrenia. *Neurosci Lett* 353:53–56.

Lijam N, Paylor R, McDonald MP, Crawley JN, Deng CX, Herrup K, et al (1997): Social interaction and sensorimotor gating abnormalities in mice lacking Dvl1. *Cell* 90:895–905.

Martin ER, Monks SA, Warren LL, Kaplan NL (2000): A test for linkage and association in general pedigrees: The pedigree disequilibrium test. *Am J Hum Genet* 67:146–154.

Miyaoka T, Seno H, Ishino H (1999): Increased expression of Wnt-1 in schizophrenic brains. *Schizophr Res* 38:1–6.

Nakajima T, Jorde LB, Ishigami T, Uemura S, Emi M, Lalouel J-M, et al (2002): Nucleotide diversity and haplotype structure of the human angiotensin gene in two populations. *Am J Hum Genet* 70:108–123.

Pleasure SJ (2001): An arrow hits the Wnt signaling pathway. *Trends Neurosci* 24:69–71.

Pritchard JK, Stephens M, Donnelly P (2000): Inference of population structure using multilocus genotype data. *Genetics* 155:945–959.

Purcell S, Cherny SS, Sham PC (2003): Genetic Power Calculator: Design of linkage and association genetic mapping studies of complex traits. *Bioinformatics* 19:149–150.

Saitou N (1995): A genetic affinity analysis of human populations. *Hum Evol* 10:17–33.

Sham PC, Curtis D (1995): An extended transmission/disequilibrium test (TDT) for multi-allele marker loci. *Ann Hum Genet* 59:323–336.

Sheldahl LC, Park M, Malbon CC, Moon RT (1999): Protein kinase C is differentially stimulated by Wnt and Frizzled homologs in a G-protein-dependent manner. *Curr Biol* 9:695–698.

Weinberger DR (1995): From neuropathology to neurodevelopment. *Lancet* 346:552–557.

Yamada K, Iwayama-Shigeno Y, Yoshida Y, Toyota T, Itokawa M, Hattori E, et al (2004): Family-based association study of schizophrenia with 444 markers and analysis of a new susceptibility locus mapped to 11q13.3. *Am J Med Genet* 127B:11–19.

Yang J, Si T, Ling Y, Ruan Y, Han Y, Wang X, et al (2003a): Association study of the human FZD3 locus with schizophrenia. *Biol Psychiatry* 54:1298–1301.

Yang JZ, Si TM, Ruan Y, Ling YS, Han YH, Wang XL, et al (2003b): Association study of neuregulin 1 gene with schizophrenia. *Mol Psychiatry* 8:706–709.

Zollner S, Wen X, Hanchard NA, Herbert MA, Ober C, Pritchard JK (2004): Evidence for extensive transmission distortion in the human genome. *Am J Hum Genet* 74:62–72.

Association Analysis of *FEZ1* Variants with Schizophrenia in Japanese Cohorts

Kazuo Yamada,* Kazuhiko Nakamura,* Yoshio Minabe,* Yoshimi Iwayama-Shigeno, Hitomi Takao, Tomoko Toyota, Eiji Hattori, Noriyoshi Takei, Yoshimoto Sekine, Katsuaki Suzuki, Yasuhide Iwata, Ko Miyoshi, Akiko Honda, Kousuke Baba, Taiichi Katayama, Masaya Tohyama, Norio Mori, and Takeo Yoshikawa

Background: *DISC1 has been suggested as a causative gene for psychoses in a large Scottish family. We recently identified FEZ1 as an interacting partner for DISC1. To investigate the role of FEZ1 in schizophrenia and bipolar disorder, case–control association analyses were conducted in Japanese cohorts.*

Methods: *We performed a mutation screen of the FEZ1 gene and detected 15 polymorphisms. Additional data on informative polymorphisms were obtained from public databases. Eight single nucleotide polymorphisms (SNPs) were analyzed in 119 bipolar disorder and 360 schizophrenic patients and age- and gender-matched control subjects. All genotypes were determined with the TaqMan assay, and selected samples were confirmed by sequencing.*

Results: *The two adjacent polymorphisms displayed a nominally significant association with schizophrenia (IVS2+1587G>A, p = .014; 396T<A or Asp123Glu, p = .024). Homozygotes with the Glu123 allele were observed in only a small portion (2%) of schizophrenia patients but not in control subjects or bipolar patients. Conversely, no SNPs displayed allelic, genotypic, or haplotypic associations with bipolar disorder.*

Conclusions: *A modest association between FEZ1 and schizophrenia suggests that this gene and the DISC1-mediated molecular pathway might play roles in the development of schizophrenia, with FEZ1 affecting only a small subset of Japanese schizophrenia patients.*

Key Words: Chromosome 11q, *DISC1*, case–control study, linkage disequilibrium block, neurodevelopment, protein kinase C

Schizophrenia is a severe and common mental illness affecting approximately 1% of the population worldwide. Etiologic evidence has suggested that the complex disorder involves multiple genetic components and environmental insults, particularly during the developmental period (Lewis and Levitt 2002; Riley and Williamson 2000).

Disrupted-in-Schizophrenia 1 (*DISC1*) on chromosome 1 was recently identified as a disrupted gene by a balanced (1;11)(q42.1; q14.3) translocation that segregated with major mental illnesses in a large Scottish family (Millar et al 2000). Although family members with altered *DISC1* showed predominantly schizophrenic symptoms, they also manifested a wide spectrum of psychiatric phenotypes, including bipolar disorder, recurrent major depression, alcoholism, and adolescent conduct disorder. Therefore, *DISC1* might be involved in psychiatric symptoms that cross diagnostic boundaries. *DISC1* shows no significant homology to any known genes, and the putative protein product has no known functional domains, making functional insights into disease pathology difficult to identify. A subsequent genetic study failed to detect any significant association between markers on *DISC1* and schizophrenia and bipolar disorder in Scottish samples (Devon et al 2001). In contrast, a Finnish schizophrenic family sample displayed haplotypic association of the gene, along with gender-dependent effects (Hennah et al 2003). *DISC1* might thus be involved in a limited subset of cases of schizophrenia and other psychoses, and different molecular components in *DICS1*-mediated signaling pathways might have a predisposition role in more general cohorts suffering from major psychoses.

Quite recently, we identified an interacting partner of DISC1, fasciculation and elongation protein zeta-*1* (FEZ1), in a yeast two-hybrid study using the C-terminal region of human DISC1 as a bait spanning the translocation break point (Miyoshi et al 2003). FEZ1 is a mammalian homologue of the *Caenorhabditis elegans* UNC-76 protein, which is involved in axonal outgrowth and fasciculation. We demonstrated that DISC1 participates in neurite extension machinery through interaction with FEZ1 (Miyoshi et al 2003). In translocation carriers in the Scottish family, *DISC1* is disrupted within intron 8, probably generating a truncated form lacking a C-terminal >200 amino acids. We also demonstrated a reduced potential for interaction with FEZ1 in this truncated DISC1. Cellular dysregulation induced by disruption of DISC1 can thus be transmitted to a downstream cascade via FEZ1. FEZ1 is located at 11q24.2, and meta-analysis has revealed that this genomic locus is a schizophrenia linkage region, although results from different sample populations are inconsistent (Lewis et al 2003). *FEZ1* might thus offer a compelling candidate for psychiatric disorders from both functional and positional perspectives.

The present study investigated genetic variants of *FEZ1* to elucidate the contribution of the gene to the risk of developing schizophrenia and bipolar disorder.

Methods and Materials

Sample Information

Subject populations in this study comprised 119 bipolar disorder patients and 360 schizophrenic patients. Two sets of

*The first three authors contributed equally to this work.

From the Laboratory for Molecular Psychiatry (KY, KN, YM, YI-S, HT, TT, EH, TY), RIKEN Brain Science Institute, Wako, Saitama; Department of Psychiatry and Neurology (KN, YM,NT, YS, KS, YI, NM), Hamamatsu University School of Medicine, Hamamatsu, Shizuoka; and Department of Anatomy and Neuroscience (KM, AH, KB, TK, MT), Osaka University Graduate School of Medicine, Osaka, Japan.

Address reprint requests to Takeo Yoshikawa, M.D., Ph.D., RIKEN Brain Science Institute, Laboratory for Molecular Psychiatry, 2–1 Hirosawa, Wako, Saitama 351–0198, Japan; E-mail: takeo@brain.riken.jp.

Received February 19, 2004; revised August 9, 2004; accepted August 26, 2004.

0006-3223/04/$30.00
doi:10.1016/j.biopsych.2004.08.015

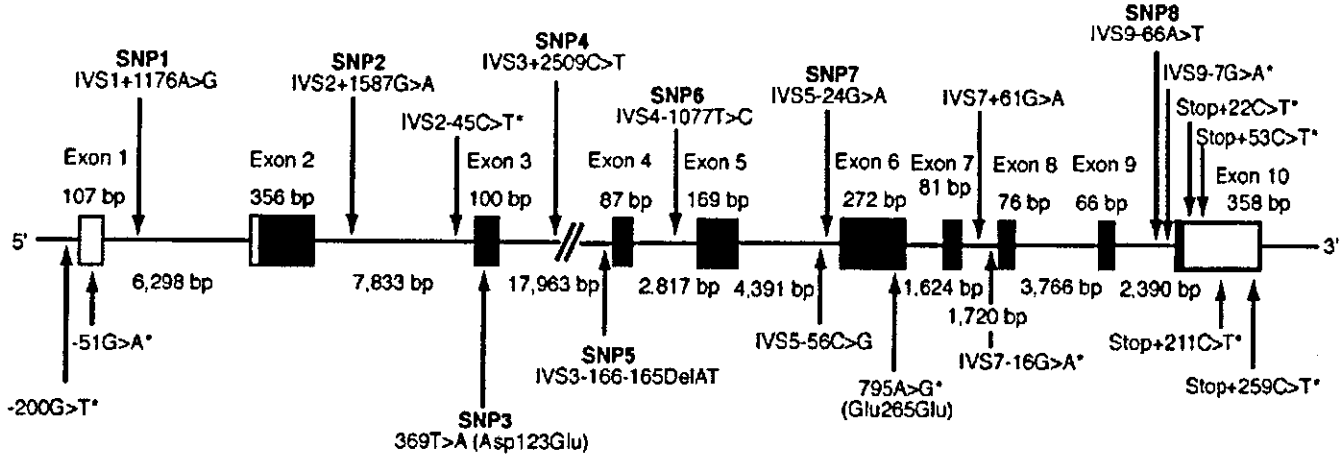

Figure 1. Genomic structure and location of polymorphic markers for *FEZ1*. Exons are denoted by boxes, with untranslated regions in open boxes and translated regions in closed boxes. Sizes of exons and introns are also shown. Novel single nucleotide polymorphisms (SNPs) identified in this study are highlighted by asterisks.

age- and gender-matched control panels were prepared, 140 for mood disorder and 360 for schizophrenia, and 99% of control samples for bipolar disorder were overlapped with those used for schizophrenia. Bipolar patients comprised 67 men and 52 women (aged 51.0 ± 12.3 years [mean ± SD]), and their control subjects comprised 70 men and 70 women (aged 51.3 ± 8.3 years). Schizophrenia patients comprised 180 men and 180 women (aged 43.7 ± 11.5 years), and their control subjects comprised 180 men and 180 women (aged 42.9 ± 12.0 years). All subjects were recruited from a geographic area located in central Japan. Best-estimate lifetime diagnosis of patients was made by direct interview with at least two experienced psychiatrists, according on DSM-IV criteria, and using all available information from medical records, hospital staff, and family informants. Control subjects were recruited from hospital staff and company employees documented to be free from psychoses and were further interviewed by experienced psychiatrists to exclude any psychiatric disorders. The ethics committee of RIKEN approved the present study, and written informed consent was obtained from all participants.

Genomic Structure, Polymorphism Search, and Marker Selection

The genomic structure of *FEZ1* was based on the University of California-Santa Cruz July 2003 draft assembly of the human genome (http://genome.ucsc.edu/) (Figure 1). All exons and splice boundaries of *FEZ1* were screened for polymorphisms by direct sequencing of polymerase chain reaction (PCR) products, generated from 30 unrelated schizophrenic patients. Primers used for PCR amplification are listed in Table 1. Direct sequencing of PCR products was performed with the BigDye terminator cycle sequencing kit (Applied Biosystems, Foster City, California) and an ABI 3700 deoxyribonucleic acid analyzer (Applied Biosystems). We also consulted multiple databases for single nucleotide polymorphism (SNP) information, including the database of Japanese Single Nucleotide Polymorphisms (http://snp.ims.u-tokyo.ac.jp/), the Celera Discovery System (http://www.celeradiscoverysystem.com/), and Entrez SNP on the National Center for Biotechnology Information web site (http://www.ncbi.nlm.nih.gov/SNP/). Heterozygosities of these SNPs were examined in 50 randomly selected Japanese subjects, and for further genetic analysis, eight SNPs that fulfilled the following criteria were chosen (Figure 1, Table 2): 1) the minor allele frequency of each SNP was >5%; 2) optimal probes and primers for the TaqMan assay (see below) could be designed; and 3) the SNP locations were evenly spread within the gene. *FEZ1* spans a genomic region of 50,474 base pairs, and the mean marker-to-marker interval was approximately 7 kilobases (kb) (each marker flanked by 3.5 kb).

Genotyping

Deoxyribonucleic acid was extracted from whole blood according to a standard protocol. Assays-by-Design SNP genotyp-

Table 1. PCR Primers Used for Screening of Nucleotide Variants in *FEZ1*

Region	Primers	Primer Sequence	Product Size (bp)
Exon 1	E01-1F	5'-ATAAAGGCAAGCCCACGTGCTC-3'	424
	E01-1R	5'-CCAGACCGTCCTGTCAGAGAAT-3'	
Exon 2	E02-1F	5'-GAGCTTCGTACTTAACCAGTGC-3'	368
	E02-1R	5'-CATTTACGAGGTCCTCCATGGA-3'	
	E02-2F	5'-TTTGAGGACCTTCGACCCTCCT-3'	429
	E02-2R	5'-TGACAGCAAGTACCAGGGCACT-3'	
Exon 3	E03-1F	5'-GAAGGCAGATGATCTCAGCCTG-3'	336
	E03-1R	5'-CAGGCATGAGCCTCCATCAACA-3'	
Exon 4	E04-1F	5'-AGTGGCCTCCCAGTCAGTATCA-3'	308
	E04-1R	5'-CACAGGCACCACATGACCACAT-3'	
Exon 5	E05-1F	5'-TCAAGCCAGAATGCCCCTCTAG-3'	353
	E05-1R	5'-ATCTGCAAGGCTCTGCTCTGAG-3'	
Exon 6	E06-1F	5'-CTGGCGTGCTCGTAAATTGTGA-3'	542
	E06-1R	5'-CTGCTCGGTAAACGTTGATGAG-3'	
Exon 7	E07-1F	5'-TAGTCACAGAACCCTGGGGCGTT-3'	285
	E07-1R	5'-TTCTGGTGAGTCACTGTCCCCCT-3'	
Exon 8	E08-1F	5'-AGTGACGTACCAGTGACCATGA-3'	325
	E08-1R	5'-TGGCCCATGTAAACCTGCTTTG-3'	
Exon 9	E09-1F	5'-CATTTCTCTCTCACTCTGGGCT-3'	291
	E09-1R	5'-CAATTGTCCCTGATTTCCCTGG-3'	
Exon 10	E10-1F	5'-CACCTGCCATTGTCTCTCCCTT-3'	346
	E10-1R	5'-CTAGCACTAGAAGCCAACGGCA-3'	
	E10-2F	5'-TCCATCACAGGGACTGCATGAG-3'	413
	E10-2R	5'-CTAAACACAGCACTGGCTGCCA-3'	

PCR, polymerase chain reaction.

Table 2. Genotypic and Allelic Distributions of *FEZ1* Polymorphisms

Markers	HUGO Nomenctature	UCSC (July 2003)[a]	Database ID[b]	Samples	Allele (%)		p Value	Genotype (%)			p Value
SNP1	IVS1 + 1176A > G	124,902,493	rs2702009		A	G		A/A	A/G	G/G	
			hCV2126433	Sc	513 (.72)	203 (.28)	1.000	181 (.51)	151 (.42)	28 (.07)	.243
				Ct	514 (.72)	202 (.28)		191 (.53)	132 (.37)	35 (.10)	
				BP	167 (.71)	69 (.28)	.491	58 (.49)	51 (.43)	9 (.08)	.819
				Ct	208 (.74)	74 (.26)		77 (.55)	52 (.37)	11 (.08)	
SNP2	IVS2 + 1587G > A	124,895,428	rs559688		G	A		G/G	G/A	A/A	
			JST072646	Sc	650 (.91)	62 (.09)	.703	301 (.85)	48 (.13)	7 (.02)	.014[c]
			hCV1061901	Ct	660 (.92)	58 (.08)		301 (.84)	58 (.16)	0 (.00)	
				BP	212 (.92)	18 (.08)	.375	98 (.85)	16 (.14)	1 (.01)	.504
				Ct	264 (.94)	18 (.08)		124 (.69)	16 (.11)	0 (.00)	
SNP3	369T > A (Asp123Glu)	124,689,124	rs597570		T	A		T/T	T/A	A/A	
			JST091863	Sc	652 (.91)	68 (.09)	.457	299 (.83)	54 (.15)	7 (.02)	.024[c]
			hCV2907168	Ct	659 (.92)	59 (.08)		300 (.84)	59 (.16)	0 (.00)	
				BP	219 (.93)	17 (.07)	.729	101 (.86)	17 (.14)	0 (.00)	.720
				Ct	282 (.94)	18 (.08)		122 (.87)	18 (.13)	0 (.00)	
SNP4	IVS3 + 2509C > T	124,886,573	hCV2907191		C	T		C/C	C/T	T/T	
				Sc	449 (.62)	271 (.38)	.549	142 (.39)	165 (.48)	53 (.15)	.587
				Ct	460 (.64)	260 (.36)		154 (.43)	152 (.42)	54 (.15)	
				BP	151 (.64)	85 (.36)	.187	45 (.38)	61 (.52)	12 (.10)	
				Ct	194 (.70)	84 (.30)		69 (.05)	56 (.40)	14 (.10)	
SNP5	IVS3 − 166-165DalAT	124,671,284	rs3217293		Ins	Del		Ins/Ins	Ins/Del	Del/Del	
			JST079190	Sc	473 (.66)	247 (.34)	.867	155 (.43)	163 (.45)	42 (.12)	.555
				Ct	477 (.66)	243 (.34)		164 (.46)	149 (.41)	47 (.13)	
				BP	152 (.64)	84 (.36)	.761	48 (.41)	56 (.47)	14 (.12)	.822
				Ct	184 (.66)	96 (.34)		63 (.45)	58 (.41)	19 (.14)	
SNP6	IVS4 − 1077T > C	124,669,291	hCV2907206		T	C		T/T	T/C	C/C	
				Sc	468 (.65)	250 (.35)	.824	153 (.43)	162 (.45)	44 (.12)	.744
				Ct	474 (.66)	248 (.34)		161 (.45)	152 (.42)	47 (.13)	
				BP	152 (.64)	84 (.36)	.926	48 (.41)	56 (.47)	14 (.12)	.607
				Ct	182 (.65)	98 (.35)		62 (.44)	58 (.41)	20 (.14)	
SNP7	IVS5 − 24G > A	124,663,678	rs2241514		G	A		G/G	G/A	A/A	
			JST013400	Sc	511 (.71)	205 (.29)	.770	188 (.52)	139 (.39)	33 (.09)	.853
			hCV2907211	Ct	519 (.72)	201 (.28)		188 (.52)	143 (.4)	29 (.08)	
				BP	170 (.72)	66 (.28)	.697	60 (.51)	50 (.42)	8 (.07)	.642
				Ct	197 (.70)	83 (.30)		71 (.51)	55 (.39)	14 (.10)	
SNP8	IVS9 − 66A > T	124,853,725	hCV2907222		A	T		A/A	A/T	T/T	
				Sc	527 (.74)	187 (.26)	.952	194 (.54)	139 (.39)	24 (.07)	.738
				Ct	528 (.74)	188 (.26)		199 (.56)	130 (.36)	28 (.08)	
				BP	172 (.74)	62 (.26)	.760	61 (.52)	50 (.43)	6 (.05)	.659
				Ct	207 (.75)	69 (.25)		78 (.57)	51 (.37)	9 (.07)	

HUGO, The Human Genome Organization; UCSC, University of California-Santa Cruz; SNP, single nucleotide polymorphism; Sc, schizophrenic patients; Ct, control subjects; BP, bipolar disorder patients.

[a] http://genome.ucac.edu/cgl-bin/hgGateway.

[b] rs number: http://www.ncbi.ntm.nih.gov/SNP/; JST number: http://snp.ims.u-tokyo.ac.jp/; hCV number: http://www.celeradiscoverysystem.com/.

[c] $p < .05$.

ing products (Applied Biosystems) were used to score SNPs according to the TaqMan assay method (Ranade et al 2001). Genotypes were determined with an ABI7900 sequence detection system instrument (Applied Biosystems) and SDS 2.0 software (Applied Biosystems). Microsatellite marker loci were amplified by PCR with fluorescently labeled primers. Polymerase chain reaction fragments were run on an ABI PRISM 3700 Genetic Analyzer (Applied Biosystems), and genotypes were determined with GeneScan 3.5.2 and Genotyper 3.6 software (Applied Biosystems). All genotypes were independently scored by YI-S and HT. Samples in which ambiguity could not be resolved after re-genotyping were omitted from subsequent analyses. Single nucleotide polymorphism 3 (Asp123Glu) showed positive association with the TaqMan assay, and this was confirmed by sequencing. We randomly selected 10 T/T homozygotes, 10 T/A heterozygotes, and all seven A/A homozygotes for either single- or double-stranded sequencing of PCR amplicons and obtained results identical to those from the TaqMan assay.

Statistical Procedures

Deviations from Hardy-Weinberg equilibrium were computed with the Arlequin program (http://lgb.unige.ch/arlequin/) (Schneider et al 2000). Normalized linkage disequilibrium (LD) coefficient (D') (Lewontin 1988) and squared correlation coefficient (r^2), a parameter indicating a degree of LD between

Table 3. SNP Markers Used for Assesment of Sample Stratification

Database ID	Location		Minor Allele Frequency	
	UCSC (2003 July) (bp)	Cytogenetic Band	Schizophrenia	Control Subjects
rs2071948	20,070,796	1p36.12	.456	.486
rs2280404	30,920,431	2p23.1	.521	.490
rs2240538	242,355,886	2q37.3	.439	.428
rs14275	4,378,153	3p26.1	.404	.400
rs2279506	120,413,529	4q26	.452	.467
rs2304865	188,221,253	4q35.2	.401	.418
rs1392468	1,797,491	5p15.33	.409	.411
rs1638212	7,061,614	7p21.3	.490	.468
rs2305944	155,225,978	7q36.3	.415	.413
rs2297082	418,706	9p24.3	.416	.411
rs917777	124,519,360	9q33.3	.438	.430
rs643966	128,179,812	11q24.3	.331	.356
rs2238013	2,045,305	12p13.33	.437	.436
rs2281231	1,541,880	16p13.3	.426	.440
rs747232	88,635,172	16q24.3	.353	.399
rs2072310	533,693	19p13.3	.494	.493
rs2278290	62,989,501	19q13.43	.434	.424
rs310672	62,939,276	20q13.33	.432	.438
rs1702405	30,913,311	21q22.11	.439	.390
rs2279632	196,188,793	3q29	.426	.427
rs2291270	169,126,860	5q35.1	.419	.452

UCSC, University of California-Santa Cruz.

markers, were calculated with COCAPHASE software (http://www.rfcgr.mrc.ac.uk/~fdudbrid/software/unphased/) (Dudbridge et al 2000). Allelic and genotypic distributions between patients and control subjects were assessed with Fisher's exact test. Haplotype frequencies were estimated with the expectation-maximization algorithm implemented in the COCAPHASE program, which was also used to evaluate haplotypic associations. Graphical overview of pairwise LD strength between markers was made with GOLD software (http://well.ox.ac.uk/asthma/GOLD/) (Abecasis and Cookson 2000).

Power for genotype analysis was derived from the noncentral χ^2 distribution, with Sample Power 2.0 software (SPSS Japan, Tokyo, Japan). The noncentrality parameter (NCP) was calculated according to the same assumption implemented in the Genetic Power Calculator (http://statgen.iop.kcl.ac.uk/gpc/) (Purcell et al 2003).

CLUSTALW (program for multiple alignments and tree-making; http://www.ddbj.nig.ac.jp/search/clustalw-e.html) (Thompson et al 1994) and TreeView 1.6.6 (program for displaying phylogenies; http://taxonomy.zoology.gla.ac.uk/rod/treeview.html) (Page 1996) software were used to depict the evolutionary history of haplotypes in a phylogram.

Assessment of Sample Stratification

For population homogeneity assessment, 21 SNPs and 41 microsatellite markers were selected genome-wide. Single nucleotide polymorphisms were selected from the databases described above. Microsatellite markers were from the Marshfield map (http://research.marshfieldclinic.org/genetics/). Detailed information on these markers is summarized in Tables 3 and 4. STRUCTURE software (http://pritch.bsd.uchicago.edu/software.html) (Pritchard et al 2000) was used to attempt to identify genetically similar diploid subpopulations by grouping individuals. In the application of this Markov chain Monte Carlo method, 1,000,000 replications were used for the burn-in period of the chain and for parameter estimation. Number of populations present in the sample (K) was unknown, so analysis was run at $K = 1, 2, 3, 4$, and 5. From these results, the best estimate of K was found by calculating posterior probabilities, $Pr(K = 1, 2, 3, 4, \text{or } 5)$, as described by Pritchard et al (2000). No evidence for stratification was identified in our samples, with a $Pr(K = 1) > .99$.

Results

Mutation Screening

Mutation screening identified 15 polymorphisms, including 10 novel variants (Figure 1 and Table 5). The heterozygosities of these SNPs are summarized in Table 5.

Case–Control Study of Bipolar Disorder

Genotypic and allelic frequencies of eight SNPs on *FEZ1* are summarized in Table 1. Genotype distributions of all SNPs in bipolar disorder patients and control subjects displayed Hardy-Weinberg equilibrium. No significant differences in allele frequencies or genotype distributions were found between bipolar disorder patients and control subjects. Significant differences in haplotype distributions were also not observed between bipolar disorder patients and control subjects (data not shown).

Case–Control Study of Schizophrenia

No significant differences in allele frequencies of the eight SNPs were obtained (Table 1). Similarly, no significant differences in haplotype distributions were noted between schizophrenia patients and control subjects (data not shown). In contrast, genotype distributions of IVS2+1587G>A (SNP2) and 396T>A (Asp123Glu in exon 3, SNP3) differed significantly between schizophrenia patients and control subjects (SNP2, nominal $p = .014$; SNP3, nominal $p = .024$; after Bonferroni correction, both p values became nonsignificant). Homozygotes with the IVS2+1587A allele of SNP2 and Glu123 allele of SNP3 were observed only in schizophrenic patients (seven patients)

Table 4. Microsatellite Markers Used for Assesment of Sample Stratification

Marker	UCSC (2003 July) (bp)		Cytogenetic Band	Heterozygosity
	Start	End		
D1S235	232,934,904	232,935,199	1q42.3	.71
D1S547	238,797,083	238,797,380	1q43	.77
D2S125	241,488,778	241,489,045	2q37.3	.82
D2S1363	227,232,127	227,232,423	2q38.3	.75
D3S1262	187,544,392	187,644,640	3q27.3	.71
D3S1744	148,413,404	148,413,762	3q24	.79
D4S1652	190,680,220	190,880,545	4q35.2	.63
D4S2366	6,449,220	6,649,489	4p18.1	.77
D4S2368	169,311,970	169,512,519	4q32.3	.69
D4S408	185,748,946	185,947,218	4q35.1	.64
D5S211	173,129,200	173,329,475	5q35.2	.84
D5S2848	26,721,029	28,921,318	5p14.1	.63
D6S1277	164,107,012	164,307,392	6q26	.72
D6S2438	154,087,047	154,287,500	6q25.2	.80
D8S1179	125,783,852	125,963,993	8q24.13	.84
D8S264	2,017,696	2,217,954	8p23.3	.83
D9S2157	131,211,512	131,411,904	9q34.2	.79
D10S1225	63,999,602	64,199,891	10q21.3	.73
D10S1230	122,307,221	122,507,499	10q28.12	.68
D10S1423	19,341,700	19,542,335	10p12.31	.70
D10S2470	91,929,173	92,129,449	10q23.31	.70
D11S912	128,061,745	128,281,860	11q24.3	.74
D11S968	133,256,028	133,456,289	11q25	.49
D12S1045	128,650,570	128,850,930	12q24.33	.75
D12S1064	89,225,736	89,426,019	12q21.33	.75
D12S391	12,241,141	12,441,471	12p13.2	.84
D13S796	105,488,858	105,887,142	13q33.3	.79
D13S800	71,572,650	71,773,041	13q22.1	.77
D14S1434	93,198,164	93,398,726	14q32.13	.75
D15S642	99,970,038	100,170,319	15q26.3	.70
D16S539	86,067,513	86,267,907	16q24.1	.77
D17S1303	10,959,851	11,160,201	17p12	.73
D17S784	78,402,240	78,802,543	17q25.3	.61
D18S481	2,958,133	3,158,372	18p11.31	.78
D18S851	48,259,389	48,459,692	18q21.1	.74
D19S589	58,398,268	58,598,602	19q13.42	.78
D19S591	2,926,844	3,127,081	19p13.3	.78
D20S171	56,393,299	58,593,701	20q13.32	.78
D20S478	37,817,396	38,017,724	20q11.23	.80
D21S1432	16,165,181	16,385,576	21q21.1	.72
D22S445	35,708,937	35,909,283	22q13.1	.87

and not in any control subjects (Table 2) (in addition, no bipolar patients displayed homozygous Glu123 alleles). Genotype distributions of all SNPs were in Hardy-Weinberg equilibrium in control subjects; however, SNP2 and SNP3 (these SNPs are in complete LD) showed deviations from Hardy-Weinberg equilibrium in schizophrenic patients. These deviations were mainly due to the overrepresentation of the rare homozygotes. Causes of these deviations are not presently clear, but the distortion might represent the close vicinity of these SNPs to real disease-causing variants in schizophrenia patients.

Linkage Disequilibrium Structure of *FEZ1* and Phylogenic Analysis of SNPs

Graphic representations of LD relationships between markers are shown in Figure 2. Squared correlation coefficients (r^2) were computed, because $r^2 \geq .1$ is an indicator for substantial LD between markers in relatively small genomic regions like that encoding *FEZ1* (Nakajima et al 2002). Linkage disequilibrium structures of the *FEZ1* genomic region were similar in the control, schizophrenia, and bipolar groups (Figure 2), and the pattern of LD was somewhat irregular: schizophrenia-associated SNPs, SNP2, and SNP3 were in complete LD to each other ($r^2 = 1.000$) but were isolated from the LD block comprising the other SNPs. That is, the gene region is composed of a double LD structure, with a small LD island surrounding SNP2 and SNP3 embedded in a larger LD block spanning from SNP1 to SNP8 (excluding SNP2 and SNP3) that covered the entire gene. These data suggest that SNP2 and SNP3 might have been introduced at a historically different age, when compared with the other SNPs. This speculation is also supported by analysis of the evolutionary history of haplotypes defined by the SNPs on *FEZ1*, which distinguishes the origin of the A-A (SNP2-SNP3) haplotype from the others (Figure 3).

Power Calculation

We calculated the genotypic power of our study on the basis of the genotype data. For the missense SNP3, the power of our

Table 5. Information of SNPs Detected by Mutation Screening

Polymorphism	Database ID	UCSC (July 2003)	Distance from Neighboring SNP (bp)	Heterozygosity
−200G > T[a]		124,903,823		.03
−51G > A[a]		124,903,674	149	.10
IVS2 − 45C > T[a]		124,889,226	14,448	.03
369T > A (Asp123Glu)	rs597570	124,889,124	102	.15
IVS5 − 56C > G	rs679182, JST013401	124,863,710	25,414	.41
IVS5 − 24G > A	rs2241514, JST013400	124,863,678	32	.40
795A > G (Glu265Glu)[a]		124,863,527	151	.03
IVS7 + 61G > A	rs11220082	124,861,617	1910	.47
IVS7 − 16G > A[a]		124,859,973	1644	.03
IVS9 − 66A > T	hCV2907222	124,853,725	6248	.38
IVS9 − 7G > A[a]		124,853,666	59	.06
Stop + 22C > T[a]		124,853,621	45	.03
Stop + 53C > T[a]		124,853,590	31	.03
Stop + 211T > C[a]		124,853,432	158	.03
Stop + 259C > T[a]		124,853,384	48	.06

SNPs, single nucleotide polymorphisms; UCSC, University of California-Santa Cruz.
[a]Novel mutations found in this study.

schizophrenic sample was based on a recessive mode of inheritance and estimated at .714 (NCP = 7.93), with a genetic relative risk (GRR) of 5.07, assuming an α value of .05 and risk allele frequency of <.1. With multiple testing (we conducted 58 statistical tests), the present sample size displayed a power of .216 with an α of .000862 (= .05/58).

Discussion

Dysfunction caused by *DISC1* is thought to account for the pathogenesis of only limited subpopulations of psychiatric illnesses (Devon et al 2001; Hennah et al 2003; Millar et al 2000). This raises the possibility that a signaling pathway involving DISC1, rather than DISC1 molecule, might play a broader role in psychiatric pathology, and each member of the DISC1 cascade might confer risk for schizophrenia, bipolar disorder, or other psychoses in individual sample populations. We demonstrated herein a genotypic association, albeit nonsignificant after correction for multiple testing, of SNP2 and SNP3 of *FEZ1* with schizophrenia in Japanese patients but not with bipolar disorder. Notably, the association was due to the fact that rare homozygotes were represented only in the schizophrenia group. This seemingly recessive mode of inheritance and the rarity of homozygotes would make detecting allelic and haplotypic associations difficult, as revealed in the current study.

In general, GRR is assumed to be 1.5–2.0 in psychiatric genetics (e.g., Risch and Merikangas 1996), where it requires a sample size of 5752 for GRR = 2.0 or 19,125 for GRR = 1.5, to obtain a statistical power of 80% under the condition of α = .05, risk allele frequency = .1, and a recessive mode of inheritance; however, the current power analysis estimated that the GRR of SNP3 was 5.07 and showed that our sample size had a moderate power (.714) to detect a difference in genotypic distributions between patients and controls subjects. But the power becomes insufficient after Bonferroni correction for multiple comparisons. This necessitates independent confirmatory studies with larger and ethnically different samples.

The meta-analysis of Lewis et al (2003) provided evidence for linkage of the *FEZ1* locus on 11q to schizophrenia; however, this result was based on positive linkage findings in small samples of extended pedigrees from homogeneous or isolated populations. This caveat might explain why the SNP2 and SNP3 homozyogtes of *FEZ1* were observed exclusively in a limited number of schizophrenic patients.

The two SNPs, SNP2 and SNP3, cannot be genetically differentiated because of tight LD between them, but SNP3 (Asp123Glu) rather than SNP2 (IVS2+1587G>A) would have functional consequences if the genomic segment harboring these SNPs confers a true predisposition. Asp123 is conserved among human, rat, and mouse species (http://kr.expasy.org/sprot/). The nematode counterpart of FEZ1, UNC-76, displays axon-targeting activity through the domain stretching across amino acids 1–197, the N-terminal third of the protein (Bloom and Horvitz 1997). Therefore, the functional effects of nonsynonymous coding polymorphisms within the human FEZ1 protein is worthy of further investigation.

The multistratum structure of the LD block for *FEZ1* suggests an historic recombination/rearrangement in this genomic region. In evolutionary terms, the SNP2 and SNP3 polymorphisms could be of a distinct origin compared with the other SNPs. If the SNP2 and SNP3 variants had been introduced into the Japanese population at an earlier stage compared with the other SNPs, then the former two genetic polymorphisms should be present in other populations, suggesting again the importance for replication studies in ethnically different samples.

FEZ1 has also been identified as a protein kinase C (PKC)-interacting protein, by showing that FEZ interacts with the NH_2-terminal variable region of PKCζ and weakly interacts with that of PKCε (Kuroda et al 1999). FEZ1 is normally localized to the plasma membrane and translocates to the cytoplasm by regulation of PKCζ activity. Interestingly, several genes related to the PKC pathway have been proposed as candidate genes for schizophrenia. First, PKC participates in Wnt signaling, and several lines of evidence have demonstrated abnormality of Wnt signaling in schizophrenia (Beasley et al 2001; Miyaoka et al 1999; Yang et al ; also see Ide et al 2004). Second, tumor necrosis factor α (TNFα), a pleiotropic proinflammatory cytokine, is a potent activator of PKCζ (Lallena et al 1999; Müller et al 1995). Significant increases in plasma concentrations of TNFα have been reported in schizophrenic patients (Kowalski et al 2001; Monteleone et al 1997; Naudin et al 1997; Theodoropoulou et al 2001).

A

	SNP1	SNP2	SNP3	SNP4	SNP5	SNP6	SNP7	SNP8
SNP1								
SNP2	0.038 (0.035) <0.022>							
SNP3	0.042 (0.035) <0.025>	1.000 (1.000) <1.000>						
SNP4	**0.653 (0.691) <0.709>**	**0.160 (0.155) <0.141>**	**0.164 (0.153) <0.140>**					
SNP5	**0.169 (0.152) <0.146>**	0.049 (0.045) <0.032>	0.053 (0.046) <0.036>	**0.252 (0.235) <0.185>**				
SNP6	**0.175 (0.155) <0.152>**	0.050 (0.046) <0.033>	0.052 (0.046) <0.034>	**0.260 (0.232) <0.193>**	1.000 (1.000) <1.000>			
SNP7	**0.112 (0.113) <0.112>**	0.037 (0.034) <0.026>	0.040 (0.035) <0.029>	**0.165 (0.166) <0.141>**	**0.754 (0.749) <0.808>**	**0.739 (0.740) <0.783>**		
SNP8	**0.811 (0.777) <0.714>**	0.034 (0.031) <0.021>	0.037 (0.032) <0.023>	**0.523 (0.525) <0.552>**	**0.182 (0.178) <0.175>**	**0.187 (0.181) <0.181>**	**0.141 (0.138) <0.141>**	
r^2	SNP1	SNP2	SNP3	SNP4	SNP5	SNP6	SNP7	SNP8

B

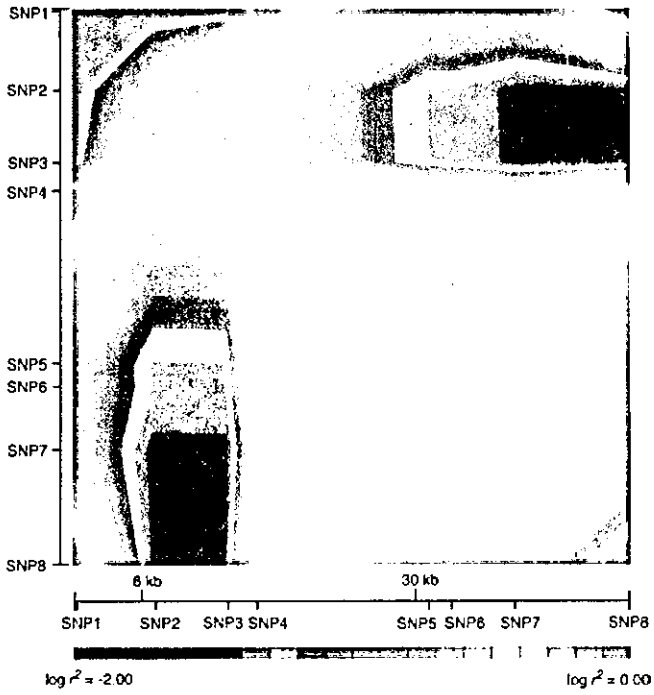

Figure 2. Linkage disequilibrium (LD) structure of *FEZ1* region. **(A)** LD: parameters between markers in control, schizophrenia (in parentheses) and bipolar (in angle brackets) groups. LD statistics, squared correlation coefficients (r^2), were calculated with COCAPHASE software. For brevity, single nucleotide polymorphisms (SNPs) are designated as SNP1–SNP8 (Table 1, Figure 1). Values of $r^2 \geq .1$ are shown in bold. **(B)** GOLD plot of color-coded LD strength between markers in the schizophrenia group. Red and yellow indicate areas of strong LD. kb, kilobase; log, logarithm.

Haplotype (SNP1-2-3-4-5-6-7-8)	Schizophrenia (%)	Control (%)
A-A-A-T-I-T-G-A	.089	.083
A-G-T-C-I-T-G-A	.286	.307
A-G-T-C-D-C-A-A	.281	.276
A-G-T-C-D-C-G-A	.060	.056
G-G-T-T-I-T-G-A	.021	.024
G-G-T-T-I-T-G-T	.263	.254

Figure 3. Phylogram and frequencies of haplotypes in *FEZ1*. For the nomenclature of single nucleotide polymorphisms (SNPs) 1–8, see Figure 1. For SNP5, "D" indicates deletion and "I" denotes insertion. The doubly underlined SNP alleles are those that show association with schizophrenia. Six different haplotypes covered 100% of the total number of haplotypes in both schizophrenia patients and control subjects.

In conclusion, we obtained data suggesting that *FEZ1* might be associated with the development of schizophrenia, specifically in a small subset of patients carrying homozygous alleles of IVS2+158A (SNP2) and Glu123 (SNP3). Given that polymorphisms were rare and deemed to be introduced by a founder from a different ethnic population, replication studies with larger sample sizes and/or other ethnic cohorts, along with detailed phenotypic inspection of SNP2 and SNP3 homozygous carriers, will be important. In addition, successive molecular elucidation of DISC1-mediated cellular mechanisms and genetic evaluation of relevant genes for DISC1/FEZ1-mediated signaling pathway in schizophrenia are warranted.

This work was supported by RIKEN BSI Funds, Research on Brain Science Funds from the Ministry of Health Labor and Welfare, and CREST funds from the Japan Science and Technology Agency, Japan.

Abecasis GR, Cookson WO (2000): GOLD-graphical overview of linkage disequilibrium. *Bioinformatics* 16:182–183.

Beasley C, Cotter D, Khan N, Pollard C, Sheppard P, Varndell I, et al (2001): Glycogen synthase kinase-3beta immunoreactivity is reduced in the prefrontal cortex in schizophrenia. *Neurosci Lett* 20:117–120.

Bloom L, Horvitz HR (1997): The *Caenorhabditis elegans* gene *unc-76* and its human homologs define a new gene family involved in axonal outgrowth and fasciculation. *Proc Natl Acad Sci U S A* 94:3414–3419.

Devon RS, Anderson S, Teague PW, Burgess P, Kipari TM, Semple CA, et al (2001): Identification of polymorphisms within *Disrupted in Schizophrenia 1* and *Disrupted in Schizophrenia* 2 and an investigation of their association with schizophrenia and bipolar affective disorder. *Psychiatr Genet* 11:71–78.

Dudbridge F, Koeleman BP, Todd JA, Clayton DG (2000): Unbiased application of the transmission/disequilibrium test to multilocus haplotypes. *Am J Hum Genet* 66:2009–2012.

Hennah W, Varilo T, Kestilä M, Paunio T, Arajärvi R, Haukka J, et al (2003): Haplotype transmission analysis provides evidence of association for DISC1 to schizophrenia and suggests sex-dependent effects. *Hum Mol Genet* 12:3151–3159.

Ide M, Yamada K, Iwayama-Shigeno Y, Iwamoto K, Takao H, et al (2004): Genetic and expression analyses of *FZD3* in Schizophrenia. *Biol Psychiatry* 56:462–465.

Kowalski J, Blada P, Kucia K, Madej A, Herman ZS (2001): Neuroleptics normalize increased release of interleukin-1b and tumor necrosis factor α from monocytes in schizophrenia. *Schizophr Res* 50:169–175.

Kuroda S, Nakagawa N, Tokunaga C, Tatematsu K, Tanizawa K (1999): Mammalian homologue of the *Caenorhabditis elegans* UNC-76 protein involved in axonal outgrowth is a protein kinase C ζ-interacting protein. *J Cell Biol* 144:403–411.

Lallena MJ, Diaz-Meco MT, Bren G, Paya CV, Moscat J (1999): Activation of IkappaB kinase beta by protein kinase C isoforms. *Mol Cell Biol* 19:2180–2188.

Lewis CM, Levinson DF, Wise LH, DeLisi LE, Straub RE, Hovatta I, et al (2003): Genome scan meta-analysis of schizophrenia and bipolar disorder, part II: Schizophrenia. *Am J Hum Genet* 73:34–48.

Lewis DA, Levitt P (2002): Schizophrenia as a disorder of neurodevelopment. *Ann Rev Neurosci* 25:409–432.

Lewontin RC (1988): On measures of gametic disequilibrium. *Genetics* 120: 849–852.

Millar JK, Wilson-Annan JC, Anderson S, Christie S, Taylor MS, Semple CAM, et al (2000): Disruption of two novel genes by a translocation co-segregating with schizophrenia. *Hum Mol Genet* 9:1415–1423.

Miyaoka T, Seno H, Ishino H (1999): Increased expression of Wnt-1 in schizophrenic brains. *Schizophr Res* 38:1–6.

Miyoshi K, Honda A, Baba K, Taniguchi M, Oono K, Fujita T, et al (2003): *Disrupted-In Schizophrenia* 1, a candidate gene for schizophrenia, participates in neurite outgrowth. *Mol Psychiatry* 8:685–694.

Monteleone P, Fabrazzo M, Tortorella A, Maj M (1997): Plasma levels of interleukin-6 and tumor necrosis factor alpha in chronic schizophrenia: Effects of clozapine treatment. *Psychiatry Res* 71:11–17.

Müller G, Ayoub M, Storz P, Rennecke J, Fabbro D, Pfizenmaier K (1995): PKC zeta is a molecular switch in signal transduction of TNF-alpha, bifunctionally regulated by ceramide and arachidonic acid. *EMBO J* 14:1961–1969.

Nakajima T, Jorde LB, Ishigami T, Uemura S, Emi M, Lalouel J-M, et al (2002): Nucleotide diversity and haplotype structure of the human angiotensin gene in two populations. *Am J Hum Genet* 70:108–123.

Naudin J, Capo C, Giusano B, Mege JL, Azorin JM (1997): A differential role for interleukin-6 and tumor necrosis factor-alpha in schizophrenia? *Schizophr Res* 26:227–233.

Page RD (1996): TreeView: An application to display phylogenetic trees on personal computers. *Comput Appl Biosci* 12:357–358.

Pritchard JK, Stephens M, Donnelly P (2000): Inference of population structure using multilocus genotype data. *Genetics* 155:945–959.

Purcell S, Cherny SS, Sham PC (2003): Genetic Power Calculator: Design of linkage and association genetic mapping studies of complex traits. *Bioinformatics* 19:149–150.

Ranade K, Chang MS, Ting CT, Pei D, Hsiao CF, Olivier M, et al (2001): High-throughput genotyping with single nucleotide polymorphisms. *Genome Res* 11:1262–1268.

Riley B, Williamson R (2000): Sane genetics for schizophrenia. *Nat Med* 6:253–255.

Risch N, Merikangas K (1996): The future of genetic studies of complex human diseases. *Science* 273:1516–1517.

Schneider S, Roessli D, Excoffier, L (2000): *Arlequin: A Software for Population Genetics Data Analysis. Ver 2.000.* Geneva: Genetics and Biometry Lab, Department of Anthropology, University of Geneva.

Theodoropoulou S, Spanakos G, Baxevanis CN, Economou M, Gritzapis AD, Papamichail MP, Stefanis CN (2001): Cytokine serum levels, autologous mixed lymphocyte reaction and surface marker analysis in never medicated and chronically medicated schizophrenic patients. *Schizophr Res* 47:13–25.

Thompson JD, Higgins DG, Gibson TJ (1994): CLUSTAL W: Improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. *Nucleic Acids Res* 22:4673–4680.

Yang J, Si T, Ling Y, Ruan Y, Han Y, Wang X, et al (2003): Association study of the human FZD3 locus with schizophrenia. *Biol Psychiatry* 54:1298–1301.

BRIEF REPORT

No Association Between the Val66Met Polymorphism of the Brain-Derived Neurotrophic Factor Gene and Bipolar Disorder in a Japanese Population: A Multicenter Study

Hiroshi Kunugi, Yoshimi Iijima, Masahiko Tatsumi, Mariko Yoshida, Ryota Hashimoto, Tadafumi Kato, Kaoru Sakamoto, Takako Fukunaga, Toshiya Inada, Tatsuyo Suzuki, Nakao Iwata, Norio Ozaki, Kazuo Yamada, and Takeo Yoshikawa

Background: *Two previous studies reported a significant association between a missense polymorphism (Val66Met) in the brain-derived neurotrophic factor (BDNF) gene and bipolar disorder; however, contradictory negative results have also been reported, necessitating further investigation.*

Methods: *We organized a multicenter study of a relatively large sample of 519 patients with bipolar disorder (according to DSM-IV criteria) and 588 control subjects matched for gender, age, and ethnicity (Japanese). Genotyping was done by polymerase chain reaction–based restriction fragment length polymorphism or direct sequencing.*

Results: *The genotype distributions and allele frequencies were similar among the patients and control subjects. Even if the possible relationships of the polymorphism with several clinical variables (i.e., bipolar I or II, presence of psychotic features, family history, and age of onset) were examined, no variable was related to the polymorphism.*

Conclusions: *The Val66Met polymorphism of the BDNF gene is unrelated to the development or clinical features of bipolar disorder, at least in a Japanese population.*

Key Words: Association study, bipolar disorder, brain-derived neurotrophic factor, genetics, single nucleotide polymorphism, susceptibility

Brain-derived neurotrophic factor (BDNF) belongs to the neurotrophin family and promotes the development, regeneration, survival, and maintenance of function of neurons (Maisonpierre et al 1991). It modulates synaptic plasticity and neurotransmitter release across multiple neurotransmitter systems, as well as the intracellular signal-transduction pathway (Thoenen 1995). Growing evidence has suggested important roles of BDNF in the pathogenesis of mood disorders and in the mechanism of action of therapeutic agents, such as mood stabilizers and antidepressants (reviewed by Duman 2002). In postmortem brains of patients with bipolar disorder, BDNF protein was reduced compared with control subjects (Knable et al 2004). Chronic electroconvulsive seizure or antidepressant drug treatments increase messenger ribonucleic acid of BDNF and its receptor, tyrosine kinase receptor B (Nibuya et al 1995). Lithium might also exert its neuroprotective effect through enhancing expression of BDNF and trkB (Hashimoto et al 2002).

The BDNF gene is, therefore, an attractive candidate gene that might cause susceptibility to bipolar disorder or influence the clinical phenotype of the illness. Indeed, at least two previous studies reported a significant association between a missense polymorphism (Val66Met; National Center for Biotechnology Information Database of Single Nucleotide Polymorphisms reference number rs6265) of the BDNF gene and bipolar disorder (Neves-Pereira et al 2002; Sklar et al 2002); however, contradictory negative results have also been reported (Hong et al 2003; Nakata et al 2003). One possible reason for this inconsistency is the lack of statistical power due to small sample size. To draw any conclusion with respect to this possible association, we organized a multicenter study in which six laboratories combined their data to ensure adequate statistical power.

Methods and Materials

Subjects

Six laboratories (National Institute of Mental Health, two laboratories of the Brain Science Institute, Showa University, Tokyo Women's Medical College, and Fujita Health University) collected deoxyribonucleic acid (DNA) samples from patients with bipolar disorder and healthy control subjects. Each institute provided DNA samples of patients and control subjects matched for gender, age, and geographic area, which yielded a combined sample of 519 patients with bipolar disorder (244 male) and 588 control subjects (287 male). Mean age (± SD) for the patients was 49.3 ± 14.3 years and for the control subjects was 48.4 ± 12.7 years. All the patients and control subjects were Japanese and biologically unrelated. Consensus diagnosis of bipolar disorder was made for each patient by at least two experienced psychiatrists according to DSM-IV criteria (American Psychiatric Association 1994) on the basis of unstructured interviews and medical records. Among the patients, 347 were diagnosed as bipolar I

From the Department of Mental Disorder Research (HK, MY, RH), National Institute of Neuroscience, National Center of Neurology and Psychiatry; Department of Psychiatry (MT), Showa University School of Medicine; Department of Psychiatry (KS, TF), Tokyo Women's Medical University School of Medicine, Tokyo; Laboratory for Molecular Dynamics of Mental Disorders (TK) and Laboratory for Molecular Psychiatry (KY, TY), Brain Science Institute, RIKEN, Saitama; Department of Psychiatry (TI, NO), Nagoya University School of Medicine; Department of Psychiatry (TS, NI), Faculty of Medicine, Fujita Health University, Aichi; and the National Institute of Mental Health (YI), National Center of Neurology and Psychiatry, Chiba, Japan.

Address reprint requests to Hiroshi Kunugi, M.D., Ph.D., National Institute of Neuroscience, National Center of Neurology and Psychiatry, Department of Mental Disorder Research, 4-1-1 Ogawahigashi, Kodaira, Tokyo 187-8502, Japan; E-mail: hkunugi@ncnp.go.jp.

Received February 27, 2004; revised June 9, 2004; accepted June 16, 2004.

0006-3223/04/$30.00
doi:10.1016/j.biopsych.2004.06.017

Table 1. Genotype Distributions and Allele Frequencies for the Val66Met Polymorphism of the BDNF Gene Among the Patients with Bipolar Disorder and Control Subjects

	Genotype Distribution				Allele Frequency	
	n	Val/Val	Val/Met	Met/Met	*n*	Val	Met
Patients						
Total	519	188 (36.2)	239 (46.1)	92 (17.7)	1038	615 (59.2)	423 (40.8)
Bipolar I	347	123 (35.4)	166 (47.8)	58 (16.7)	694	412 (59.4)	282 (40.6)
Bipolar II	172	65 (37.8)	73 (42.4)	34 (19.8)	344	203 (59.0)	141 (41.0)
Control subjects	588	216 (36.7)	270 (45.9)	102 (17.3)	1176	702 (59.7)	474 (40.3)

Values in parentheses are percentages. Genotypewise comparisons: total patients vs. control subjects: χ^2 (2) = .0, p = .98; bipolar I vs. control subjects: χ^2 (2) = .3, p = .86; bipolar II vs. control subjects: χ^2 (2) = .8, p = .69. Allelewise comparisons: total patients vs. control subjects: χ^2 (1) = .0, p = .83; bipolar I vs. control subjects: χ^2 (1) = .0, p = .96; bipolar II vs. control subjects: χ^2 (1) = .0, p = .94.

and the remaining 172 as bipolar II. Control subjects were healthy volunteers who had no current or past contact with psychiatric services. The control subjects were recruited from the hospital staffs and their associates at each institution who showed good social functioning and reported themselves to be in good health. They were interviewed, and those individuals who had current or past contact with psychiatric services were excluded. Written informed consent for participation in the study was obtained from all subjects. The study protocol was approved by the institutional ethics committees.

Methods

Venous blood was drawn, and genomic DNA was extracted according to standard procedures. Genotyping was performed according to Neves-Pereira et al (2003). Briefly, the polymorphic site was amplified by polymerase chain reaction (PCR) and then digested with a restriction enzyme, Eco72I. The digested PCR products were visualized with gel electrophoresis and subsequent ethidium bromide staining. Genotyping for a portion of subjects was done by direct sequencing of PCR products encompassing the polymorphic site with an autosequencer (CEQ 8000; Beckman Coulter, Fullerton, California). Genotype data were read blind to the case–control status.

To examine the possible relationships of the Val66Met polymorphism with clinical variables, information on age of onset, family history, and presence of psychotic features (i.e., current or past episode with delusions or hallucinations) was obtained. We defined positive family history as having at least one first-degree relative with a history of contact with psychiatric services with a diagnosis of mood disorder or who was a suicide victim. Individuals with ambiguous clinical data were excluded from statistical analyses.

The presence of Hardy-Weinberg equilibrium for the genotype distributions in the patients and control subjects was examined with the χ^2 test for goodness of fit. The differences in the genotype and allele distributions between patients and control subjects were examined with the χ^2 test for independence. The possible relationships between the polymorphism and clinical variables were examined with the χ^2 test for independence or analysis of covariance (ANCOVA) within the patient group. All p values reported are two-tailed.

Results

Genotype and allele distributions of the Val66Met polymorphism in the patients and control subjects are shown in Table 1. The genotype distributions in the two groups were both in Hardy-Weinberg equilibrium [patients: $\chi^2(1) = 1.1$, $p = .29$; control subjects: $\chi^2(1) = 1.2$, $p = .27$]. The genotype and allele distributions for the patients were quite similar to those for the control group (see Table 1). The genotype and allele distributions of the patients with bipolar I and those with bipolar II were also similar.

When relationships between genotype and clinical variables were examined, genotype and allele distributions were not different according to presence of psychotic features (frequency of the Val66 allele for psychotic patients: .567; for nonpsychotic patients: .579) or family history (positive family history: .602; negative: .603). Age of onset was also similar, irrespective of the genotype (Val/Val: 35.3 ± 13.5 years; Val/Met: 37.7 ± 14.6 years; Met/Met: 36.3 ± 14.0 years). Even when ANCOVA controlling for age and gender was performed, there was no significant difference in age of onset across the three genotypic groups [$F(2) = .99$, $p = .37$].

Discussion

We tried to replicate the studies of Sklar et al (2002) and Neves-Pereira et al (2002), who found a significant association between the Val66Met polymorphism of the BDNF gene with bipolar disorder. They reported excess transmission of the Val66 allele to the patients in their family-based association studies. Contrary to these findings, the genotype and allele frequencies among the patients and control subjects were similar in our sample, which is in turn consistent with more recent studies (Hong et al 2003; Nakata et al 2003), suggesting that the Val66Met polymorphism of the BDNF gene is unrelated to the development of bipolar disorder in our sample. Because our study had adequate statistical power (more than 90% to detect an odds ratio of 1.33 or more in allelic association; power analysis was performed according to Armitage and Berry 1994), the potential type II error due to lack of statistical power is unlikely. One possible explanation for this inconsistency might be a differential effect of the polymorphism depending on ethnicity, given that the majority of the subjects of Sklar et al (2002) and Neves-Pereira et al (2002) were Caucasian, whereas those of Hong et al (2003), Nakata et al (2003), and in our study were Asian. Alternatively, the positive results of Sklar et al (2002) and Neves-Pereira et al (2002) might have arisen by chance.

Concerning the possible effect of the polymorphism on clinical features, Rybakowski et al (2003) reported an earlier age of onset in Val/Val than Val/Met genotype (27 years vs. 38 years) among patients with bipolar disorder. They also found that the performance in all domains of the Wisconsin Card Sorting Test was significantly better for bipolar patients with Val/Val than for those with Val/Met genotype, suggesting a role of the Val66Met polymorphism in prefrontal cognitive function in bipolar disorder. This accords with the findings of Egan et al (2003), who

reported that the Met66 allele was associated with lower activity-dependent secretion of BDNF and poorer human memory and hippocampal function; however, we could not find any significant effect of the genotype on clinical variables of age of onset, subtype (bipolar I or II), psychotic features, or family history. Hong et al (2003) also failed to find significant difference in age of onset or suicidal history across genotypic groups in their Chinese subjects with bipolar disorder.

In conclusion, our results, together with previous two studies (Hong et al 2003; Nakata et al 2003), suggest that the Val66Met polymorphism of the BDNF gene is unrelated to the development or clinical features of bipolar disorder at least in Asian populations; however, the possibility remains that other variants of the BDNF gene might be associated with bipolar disorder in Asian populations, which requires further investigation.

This work was supported in part by the Health and Labor Science Research Grants for Psychiatric and Neurologic Diseases and Mental Health from the Ministry of Health, Labor and Welfare (HK).

American Psychiatric Association (1994): *Diagnostic and Statistical Manual of Mental Disorders*, 4th ed. Washington, DC: American Psychiatric Association.

Armitage P, Berry G (1994): *Statistical Methods in Medical Research*, 3rd ed. Oxford: Blackwell Science.

Duman RS (2002): Synaptic plasticity and mood disorders. *Mol Psychiatry* 7(suppl 1):S29–S34.

Egan MF, Kojima M, Callicott JH, Goldberg TE, Kolachana BS, Bertolino A, et al (2003): The BDNF val66 met polymorphism affects activity-dependent secretion of BDNF and human memory and hippocampal function. *Cell* 112:257–269.

Hashimoto R, Takei N, Shimazu K, Christ L, Lu B, Chuang DM (2002): Lithium induces brain-derived neurotrophic factor and activates TrkB in rodent cortical neurons: An essential step for neuroprotection against glutamate excitotoxicity. *Neuropharmacology* 43:1173–1179.

Hong CJ, Huo SJ, Yen FC, Tung CL, Pan GM, Tsai SJ (2003): Association study of a brain-derived neurotrophic-factor genetic polymorphism and mood disorders, age of onset and suicidal behavior. *Neuropsychobiology* 48:186–189.

Knable MB, Barci BM, Webster MJ, Meador-Woodruff J, Torrey EF (2004): Molecular abnormalities of the hippocampus in severe psychiatric illness: Postmortem findings from the Stanley Neuropathology Consortium. *Mol Psychiatry* 9:609–620.

Maisonpierre PC, Le Beau MM, Espinosa RIII, Ip NY, Belluscio L, de la Monte SM, et al (1991): Human and rat brain-derived neurotrophic factor and neurotrophin-3: Gene structures, distributions and chromosomal localizations. *Genomics* 10:558–568.

Nakata K, Ujike H, Sakai A, Uchida N, Nomura A, Imamura T, et al (2003): Association study of the brain-derived neurotrophic factor (BDNF) gene with bipolar disorder. *Neurosci Lett* 337:17–20.

Neves-Pereira M, Mundo E, Muglia P, King N, Macciardi F, Kennedy JL (2002): The brain-derived neurotrophic factor gene confers susceptibility to bipolar disorder: Evidence from a family-based association study. *Am J Hum Genet* 71:651–655.

Nibuya M, Morinobu S, Duman RS (1995): Regulation of BDNF and trkB mRNA in rat brain by chronic electroconvulsive seizure and antidepressant drug treatments. *J Neurosci* 15:7539–7547.

Rybakowski JK, Borkowska A, Czerski PM, Skibinska M, Hauser J (2003): Polymorphism of the brain-derived neurotrophic factor gene and performance on a cognitive prefrontal test in bipolar patients. *Bipolar Disord* 5:468–472.

Sklar P, Gabriel SB, McInnis MG, Bennett P, Lim YM, Tsan G, et al (2002): Family-based association study of 76 candidate genes in bipolar disorder: BDNF is a potential risk locus. *Mol Psychiatry* 7:579–593.

Thoenen H (1995): Neurotrophins and neuronal plasticity. *Science* 270:593–598.

J Med Dent Sci 2004; 51: 121–128

Original Article

Case-control association study of human netrin G1 gene in Japanese schizophrenia

Masayuki Fukasawa[1,2], Mika Aoki[1,2], Kazuo Yamada[1], Yoshimi Iwayama-Shigeno[1], Hitomi Takao[1], Joanne Meerabux[1], Tomoko Toyota[1,2], Toru Nishikawa[2] and Takeo Yoshikawa[1]

1) Laboratory for Molecular Psychiatry, RIKEN Brain Science Institute, Saitama, Japan
2) Section of Psychiatry and Behavioral Sciences, Tokyo Medical and Dental University Graduate School, Tokyo, Japan

The exact etiology of schizophrenia remains undetermined but accumulating evidence suggests that disturbances in neurodevelopment may represent one contributory factor. Netrin G1, a recently cloned gene from the mouse, has been shown to play a potential role in the formation of neural circuitry. To determine whether this gene is involved in the development of psychosis, we performed a genetic association study of human netrin G1 gene in schizophrenia. First, we determined the human genomic structure of netrin G1 by direct comparisons between cDNA and genome sequences, and by database searches. For the subsequent examination of heterozygosity, we selected 10 single nucleotide polymorphisms (SNPs) for an association test in case (n = 180) and control (n = 180) samples. Among these SNPs, IVS8-1467C>T showed significant allelic association (nominal $P = 0.020$) with disease. This SNP is located in a haplotype block of ~40 kb and haplotypes in this block also displayed significant association (most significant $P = 0.017$). These findings suggest that netrin G1 or a nearby gene may contribute to the overall genetic risk for schizophrenia.

Correspondence to: Takeo Yoshikawa, M.D., Ph.D.
Laboratory for Molecular Psychiatry
RIKEN Brain Science Institute
2-1 Hirosawa, Wako-city, Saitama 351-0198, Japan
Tel: +81(Japan)-48-467-5968
Fax: +81(Japan)-48-467-7462
E-mail: takeo@brain.riken.go.jp
Received February 6; Accepted March 19, 2004

Key words: netrin family, laminet 1, axon guidance, haplotype, linkage disequilibrium

Introduction

Schizophrenia is a common and devastating mental disorder of unknown etiology. Multiple factors including risk-conferring genes and undefined environmental variables are thought to contribute to overall susceptibility.[1] One etiological hypothesis is that neurodevelopmental abnormalities are at least partially involved in the manifestation of schizophrenia. This assertion is supported by a range of epidemiological, clinical and neurobiological evidence.[2]

The developing nervous system is dependent on the actions of various secreted factors and membrane proteins that allow neuronal axons to find their correct targets. The proteins that provide these cues include netrins, ephrins, semaphorins and slits.[3] Classical netrins identified as laminin like molecules that direct migration in *Caenorhabditis elegans* are soluble secreted proteins that provide bifunctional axon guidance signals that can mediate either attraction or repulsion.[4] Three classical netrin molecules (1, 2 and 3) have been characterized in vertebrates.[5] The gene family is structurally related to the short arms of the laminin γ chain, comprising a laminin VI domain, three LE repeats, similar to the laminin V domain and a positively charged heparin-binding carboxyl domain.[6]

Recently, netrin G1 (also called laminet 1) has been identified in the mouse.[7] Its predicted domain structure resembles that of the laminin β chain and the protein is

linked to the plasma membrane by a glycosyl phosphatidyl-inositol (GPI) lipid anchor, an important feature that distinguishes them from classical netrins.[7,8] Additionally, netrin G1 does not bind to receptors for classical netrins, nor does it attract circumferentially growing axons from the cerebellar plate in explant extracts.[7] No orthologues for netrin G1 gene have been found in the *C. elegans* or *D. melanogaster*. These observations suggest that netrin G1 may play an as yet, undetermined role in cell architecture that is unique to vertebrates.

Based on the potential relevance of netrin G1 to neurodevelopment, we performed a genetic analysis of this gene in schizophrenia.

Material and Methods

Subjects

Schizophrenic samples were composed of 90 males (mean age, 40.3±8.6 years) and 90 females (mean age, 47.1±13.0 years). All patients were diagnosed according to the criteria of the Diagnostic and Statistical Manual of Mental Disorders, Fourth Edition (DSM-IV) for schizophrenia, to give a best-estimate lifetime diagnosis with consensus from at least two experienced psychiatrists (Yamada K, Toyota T and Yoshikawa T). The interview parameters included those described in the Structured Clinical Interview For DSM-IV Axis I Disorders. All available medical records and family informant reports were also taken into consideration. Controls comprising 90 males (mean age, 39.3±11.5 years) and 90 females (mean age, 46.9±11.9 years), were recruited from hospital staff and company employees documented to be free from psychoses. All of our samples were collected from central Japan.

The present study was approved by the Ethics Committees of RIKEN and Tokyo Medical and Dental University, and all participants provided written informed consent.

Determination of genomic organization of netrin G1 gene

A mouse netrin G1 cDNA sequence NM_030699 and a human EST (expressed sequence tag) clone KIAA0976 sequence (NM_014917) were compared to human BAC clones forming the contig NT_029860 using BLAST, to determine the intron/exon structure of the human netrin G1 gene. This led to the identification of 10 exons, with translation starting within exon 2 (Fig. 1). The UCSC April 2003 draft assembly of the human

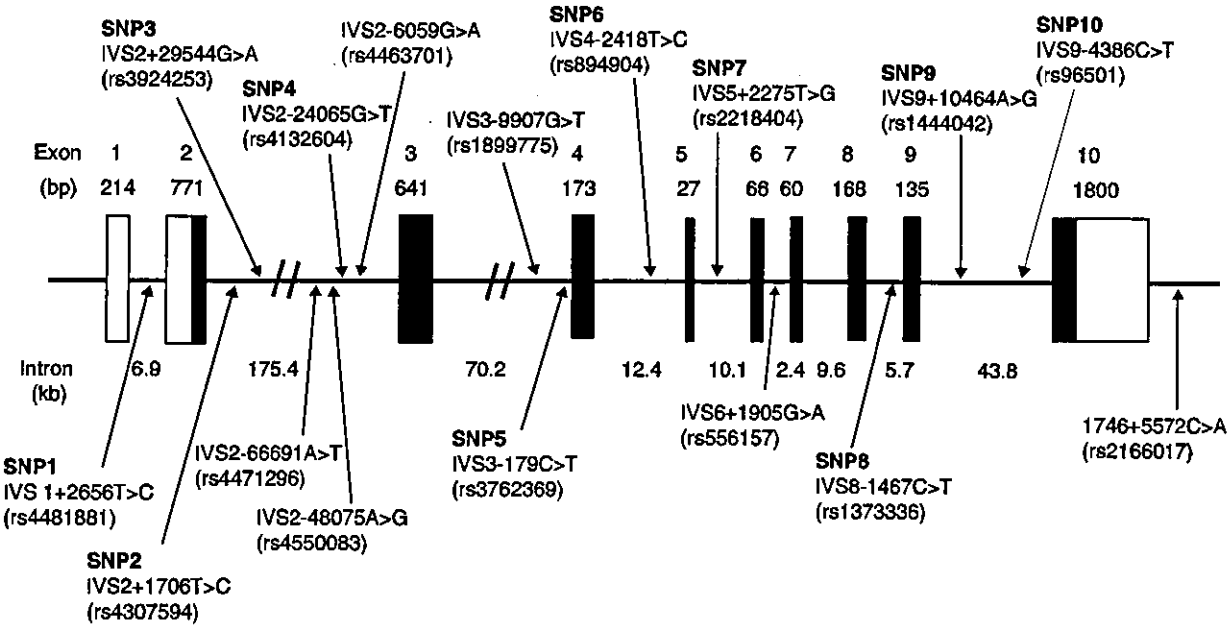

Fig. 1. Genomic structure and locations of polymorphic sites for the human netrin G1 gene. Exons are denoted by boxes, with untranslated regions in open boxes and translated regions in closed boxes. The sizes of exons (bp) and introns (kb) are also shown. The rs number of each SNP is the NCBI SNP cluster ID from the dbSNP database (http://www.ncbi.nlm.nih.gov/SNP/).

genome (UCSC Genome Bioinformatics web site, http://genome.ucsc.edu/) included only exons 1 to 5 in its gene prediction program. "A" from the ATG initiation codon was considered at +1.

Single nucleotide polymorphisms (SNPs) search and genotyping

We consulted the JSNP database (http://snp.ims.u-tokyo.ac.jp/) and The SNP Consortium Ltd database (http://snp.cshl.org/) to find polymorphisms within the netrin G1 gene, and identified a total of 16 SNPs (Fig. 1). We first genotyped these SNPs using 40 randomly chosen schizophrenic samples, and direct sequencing of PCR products (the SNPs located in exons and nearby introns) or the TaqMan method[9] (Applied Biosystems, Foster City, California, US) (the SNPs located in deep introns). The primers used for PCR amplification are shown in Table 1. Sequencing of PCR products was performed using the BigDye Terminator Cycle Sequencing FS Ready Reaction kit (Applied Biosystems) and the ABI 3700 Genetic Analyzer (Applied Biosystems). The SNPs that were not polymorphic in the 40 samples were excluded from further genetic analyses. These included IVS2-66691A>T (rs4471296, http://www.ncbi.nlm.nih.gov/SNP/), IVS2-48075A>G (rs4550083), IVS2-6059G>A (rs4463701), IVS3-9907G> T (rs1899775), IVS6+1905G> A (rs556157) and 1746+5572C>A (rs2166017) (Fig. 1). The remaining 10 variants were genotyped in all samples using the TAQman method, that utilizes the 5'-exonuclease activity of the Taq polymerase in combination of PCR and competitive hybridization.[9] Probes and primers were designed using the Assays-by-Design File Builder v2.0 software and the Primer Express software (Applied Biosystems) (Table 2). PCR reactions were performed using an ABI 9700 thermocycler, and fluorescence-based genotyping was conducted using an ABI 7900 sequence detection system and SDS v2.0 software (Applied Biosystems). The samples with ambiguous genotypes were not used in statistical analyses.

Statistical analysis

Deviation from Hardy-Weinberg equilibrium was

Table 1. PCR Primers used to examine nucleotide variants in the *NTNG1*

Region	Primers (F, forward; R, reverse)	Product size (bp)	3' end of primer
Exon 2	(F) 5'-TGCTTTATATTGCATCAGACCTC-3'	480	-603 (Intron 1)
	(R) 5'-GACCTCAAAGCAGATCCCAAAA-3'		-146 (exon 2)
	(F) 5'-AGTATGTTAGGCTTCCACCAA-3'	566	-218 (exon 2)
	(R) 5'-GTCTTCACACTCACCACATC-3'		+328 (Intron 2)
Exon 3	(F) 5'-TAGGGCAAATAAAAATGA-3'	584	+175568 (Intron 2)
	(R) 5'-AAAAACGCGAACCTGTC-3'		+176134 (exon 3)
	(F) 5'-ACGAACATGGCAGCCCTATCAG-3'	529	+175966 (exon 3)
	(R) 5'-AATGCCTTCAGAACCTACT-3'		+176473 (Intron 3)
Exon 4	(F) 5'-GGCCTGCAAATCTATCTCTTACTA-3'	511	+246439 (Intron 3)
	(R) 5'-GATGACTGAATTTTACTGAT-3'		+246877 (Intron 4)
Exon 5	(F) 5'-TGCACCTGTATTTTGTGTGTGTGC-3'	283	+258962 (Intron 4)
	(R) 5'-CCTATTACATCAGAAATGGACACT-3'		+259221 (Intron 5)
Exon 6	(F) 5'-AATTGCTTGCTCTTGTT-3'	347	+269832 (Intron 5)
	(R) 5'-TTTCAAAGACATAGCATTCAT-3'		+270162 (Intron 6)
Exon 7	(F) 5'-CTTAATTTAGGGCTACTTT T CA-3'	254	+272404 (Intron 6)
	(R) 5'-TACACTTCACAGATATTCCAGATT-3'		+272636 (Intron 7)
Exon 8	(F) 5'-ATGCCATTCCACCGTCTTT-3'	406	+282063 (Intron 7)
	(R) 5'-AGGATATTTTCTACATTGAG-3'		+282450 (Intron 8)
Exon 9	(F) 5'-TCATTAATGGACATCTTT-3'	352	+287966 (Intron 8)
	(R) 5'-GGATCTTTTTCTGCTCTGA-3'		+288300 (Intron 9)
Exon 10	(F) 5'-GGCTGAAAACATGATGTACCAGATG-3'	453	+331947 (Intron 9)
	(R) 5'-AGGCCTTCTTAGTTTGTACACTGTC-3'		+332375 (exon 10)

Nucleotide positions are counted from A of the start codons on the genomic stretches of *NTNG1* (GenBank accession No. NM_014917).

Table 2. TaqMan primer and probe sequences used to examine nucleotide variants in the *NTNG1*

Marker [a]	Primer (F, forward; R, reverse)	3' end of primer [b]	Reporter probe sequence (V, VIC label; F, FAM label) [c]
SNP1	(F) CTCTGAACTTCCCTGCATGAGAATTAA	-4826 (Intron 1)	(V) AAGAAATCTGGAATTTAA
	(R) GAGGATGGGATAATACCCAAAACTATTTCA	-4768 (Intron 1)	(F) AAGAAATCTGGAGTTTAA
SNP2	(F)GCTCTGTATATTGGTTTACATGACACTTGT	+1944 (Intron 2)	(V) CAGAAGGGACATGTTGT
	(R)CCCTGAGAAAGGAAATTTGCTATTTTACTTAGA	+1985 (Intron 2)	(F) CAGAAGGGACGTGTTGT
SNP3	(F) CATGCCATCTTTTACAAGTAACTCCTATCT	+29765 (Intron2)	(V) ATTTGCCCCGTTTCT
	(R) CCTCATAAACATGTGGCTATAATGAAGGA	+29814 (Intron2)	(F) TTTGCCCCATTTCT
SNP4	(F)GAAGAGTGTTGCTAAGCAGAGGTT	+151610 (Intron2)	(V) CACACTGGCAGCTTG
	(R) AGCCAACCACATCTATAGGCAAAAT	+151636 (Intron2)	(F) CACACTGTCAGCTTG
SNP5	(F) CCCACTGACTTGACTGCTATTCG	+246328 (Intron3)	(V) CCCTGCCCCCTGCAA
	(R) GCCCTCAGAATCTTCCCCAAAAG	+246393 (Intron3)	(F) CCCTGCCCTCTGCAA
SNP6	(F) TTTTAAAATATAGTAGTGGACATCCCTTGGTG	+256659 (Intron4)	(V) CTTTGAAGTTGAAAATATCTGAA
	(R) GGGAATTGCTTAGAAAAGATTTTTAAACTGC	+256699 (Intron4)	(F) TTTGAAGTTGAAAATGTCTGAA
SNP7	(F) AGGGCAAGAATTCACACGTAGAC	+261371 (Intron5)	(V) CACTGCCTGTTCCAG
	(R) GCACCTTGAAAACCCCATTCC	+261397 (Intron5)	(F) CTGCCGGTTCCAG
SNP8	(F) CATTGGTGATTTATTGTCAACTTACACACT	+286572 (Intron8)	(V) AAATTTGTACCTATATCGAAACT
	(R) CTTTTAATGTTTGTCTGTACATAGGCACA	+286624 (Intron8)	(F) TTGTACCTATATCAAAACT
SNP9	(F) CCCAATGGACTTAGCCTATGTGTTA	+298665 (Intron9)	(V) TCATGAAAATCAATAATATG
	(R) TGCATTCAACATCTGTGGGAACAT	+298684 (Intron9)	(F) TCATGAAAATCAGTAATATG
SNP10	(F) GGATCACTGCATTTTTCTGACATTGT	+327627 (Intron9)	(V)AAGACCATAAAGGATGCTG
	(R) CAGCATTTAGGCCATTGGAAGAGA	+327641 (Intron9)	(F)ACCATAAAGAATGCTG

[a] For SNP numbers, see Fig. 1.

[b] Nucleotide positions are counted from A of the start codon on the genomic stretches of *NTNG1* (GenBank accession No. NM_014917).

[c] Underline shows a polymophic site.

examined using the χ^2 test. Differences in genotype and allele frequency were evaluated using Fisher's exact test. Linkage disequilibrium (LD) statistics were calculated using COCAPHASE[10] (http://www.hgmp.mrc.ac.uk/~fdudbrid/software/). Estimation and comparison of haplotype frequencies were also made using COCAPHASE. Graphical overview of pair-wise LD strength between markers was made using GOLD software[11] (http://www.well.ox.ac.uk/asthma/GOLD/). Power calculations were performed using the Power Calculator (http://calculators.stat.ucla.edu/powercalc/).

Results

The alignment of cDNA and EST sequences with genomic sequence revealed that the human netrin G1 gene is comprised of 10 exons (Fig. 1) located on chromosome 1p13.3 (http://genome.ucsc.edu/). A database search for polymorphisms detected only intronic SNPs within the gene, and we selected 16 roughly equidistant SNPs. Then we examined the heterozygosity of each SNP using 40 unrelated DNAs and excluded six SNPs for further analyses based on their low heterozygosity (frequencies of minor alleles < 1%). The remaining SNPs were designated SNP1-10 (Fig. 1), and were genotyped in 180 schizophrenics and 180 age- and gender-matched controls.

All genotyped polymorphisms were in Hardy-Weinberg equilibrium in both case and control samples (Table 3). Of the 10 SNPs, SNP8 (IVS8-1476C>T) (NCBI dbSNP accession No. rs1373336, http://www.ncbi.nlm.nih.gov/SNP/) displayed a marginally significantly different genotypic distribution between patients with schizophrenia and control subjects ($P = 0.057$; Table 3). Allelic distribution of SNP8 showed a significant deviation in schizophrenics compared to controls: the C allele was over-represented in

Table 3. Genotypic and allelic distributions of the netrin G1 gene polymorphisms

Polymorphism	n [a]	Genotype counts (frequency)			HWE [b]	*P* value [c]	Allele counts (frequency)		*P* value [c]
SNP1: IVS1+2656T>C		T/T	T/C	C/C			T	C	
Schizophrenia	179	66 (0.37)	92 (0.51)	21 (0.12)	0.193	0.748	224 (0.63)	134 (0.37)	0.938
Control	173	66 (0.38)	83 (0.48)	24 (0.14)	0.796		215 (0.62)	131 (0.38)	
SNP2: IVS2+1706T>C		T/T	T/C	C/C			T	C	
Schizophrenia	178	76 (0.43)	88 (0.49)	14 (0.08)	0.095	0.186	240 (0.67)	116 (0.33)	0.384
Control	175	74 (0.42)	77 (0.44)	24 (0.14)	0.581		225 (0.64)	125 (0.36)	
SNP3: IVS2+29544G>A		A/A	A/G	G/G			A	G	
Schizophrenia	180	120 (0.67)	54 (0.30)	6 (0.03)	0.980	0.727	294 (0.82)	66 (0.18)	1.000
Control	179	117 (0.65)	58 (0.32)	4 (0.02)	0.300		292 (0.82)	66 (0.18)	
SNP4: IVS2−24065G>T		G/G	G/T	T/T			G	T	
Schizophrenia	179	61 (0.34)	81 (0.45)	37 (0.21)	0.294	0.431	203 (0.57)	155 (0.43)	0.764
Control	180	53 (0.29)	94 (0.52)	33 (0.18)	0.440		200 (0.56)	160 (0.44)	
SNP5: IVS3−179C>T		C/C	C/T	T/T			C	T	
Schizophrenia	180	115 (0.64)	55 (0.31)	10 (0.06)	0.323	0.407	285 (0.79)	75 (0.21)	0.927
Control	179	109 (0.61)	64 (0.36)	6 (0.03)	0.358		282 (0.79)	76 (0.21)	
SNP6: IVS4−2418T>C		T/T	T/C	C/C			T	C	
Schizophrenia	179	115 (0.64)	54 (0.30)	10 (0.06)	0.284	0.392	284 (0.79)	74 (0.21)	0.927
Control	180	110 (0.61)	64 (0.36)	6 (0.03)	0.365		284 (0.79)	76 (0.21)	
SNP7: IVS5+2275T>G		G/G	G/T	T/T			G	T	
Schizophrenia	179	115 (0.64)	54 (0.30)	10 (0.06)	0.284	0.359	284 (0.79)	74 (0.21)	0.855
Control	179	108 (0.60)	65 (0.36)	6 (0.03)	0.313		281 (0.78)	77 (0.22)	
SNP8: IVS8−1467C>T		C/C	C/T	T/T			C	T	
Schizophrenia	178	78 (0.44)	82 (0.46)	18 (0.10)	0.599	0.057	238 (0.67)	118 (0.33)	0.020
Control	176	59 (0.34)	87 (0.49)	30 (0.17)	0.830		205 (0.58)	147 (0.42)	
SNP9: IVS9+10464A>G		A/A	A/G	G/G			A	G	
Schizophrenia	180	79 (0.44)	73 (0.41)	28 (0.16)	0.113	0.208	231 (0.64)	129 (0.36)	0.132
Control	180	88 (0.49)	75 (0.42)	17 (0.09)	0.860		251 (0.70)	109 (0.30)	
SNP10: IVS9−4386C>T		T/T	T/C	C/C			T	C	
Schizophrenia	180	121 (0.67)	54 (0.30)	5 (0.03)	0.725	0.431	296 (0.82)	64 (0.18)	0.344
Control	179	115 (0.64)	54 (0.30)	10 (0.06)	0.284		284 (0.79)	74 (0.21)	

[a] The samples with ambigous genotypes were not included.

[b] *P* values for Hardy-Weinberg equilibrium are denoted.

[c] Differences in genotypic and allelic distributions were evaulated by Fisher's exact test.

schizophrenia (nominal $P = 0.020$, odds ratio = 1.44, 95% confidence interval = 1.06−1.96) (Table 3). After Bonferroni correction for the multiple testing of 10 SNPs, the deviation was no longer significant.

Next, we examined pair-wise linkage disequilibrium (LD) between markers. D' (normalized D) and r^2 (squared correlation coefficient) values were computed in controls. Both LD measures take values between 0 (lack of LD) and 1 (complete LD). LD relationships between SNPs are shown in Table 4 and Fig. 2. SNPs5-9 were in the same LD block using the two measures. The polymorphism (SNP8) associated with schizophrenia was located in this LD block. We examined two and three SNP-based haplotypic associations in a sliding manner, using the 10 polymorphisms that spanned netrin G1 gene (Fig. 3). The combinations of SNP7-SNP8 and SNP6-SNP7-SNP8 showed significant associations with schizophrenia (global $P = 0.017$ and 0.021, respectively). For two SNP haplotypes, the haplotype G (SNP7)-C (SNP8) was significantly more frequent in schizophrenia (frequency = 0.461) than in control group (0.362) ($P = 0.007$, odds ratio = 1.51, 95% C.I. = 1.12−2.03). For three SNP haplotypes, the haplotype T (SNP6)-G (SNP7)-C (SNP8) was significantly more over-represented in schizophrenia (0.458) than in controls (0.364) ($P = 0.010$, odds ratio = 1.46, 95% C.I. = 1.08−1.98). The results of these haplotypic associations were consistent with those of gene LD structure and allelic (genotypic) association of SNP8 with schizophrenia.

Table 4. Pairwise marker-to-marker LD statistics of *NTNG1*

Marker*	SNP1	SNP2	SNP3	SNP4	SNP5	SNP6	SNP7	SNP8	SNP9	SNP10
SNP1		**1.000**	0.069	**0.671**	0.038	0.038	0.038	0.214	0.114	0.013
SNP2	**0.260**		0.150	0.231	0.055	0.055	0.055	0.104	0.074	0.153
SNP3	0.002	0.002		**0.670**	0.006	0.006	0.006	0.084	0.020	0.084
SNP4	0.041	0.019	**0.101**		**0.319**	**0.319**	**0.319**	0.057	0.238	0.083
SNP5	0.000	0.000	0.000	0.027		**1.000**	**1.000**	**1.000**	**0.901**	0.143
SNP6	0.000	0.000	0.000	0.027	**1.000**		**1.000**	**1.000**	**0.901**	0.143
SNP7	0.000	0.000	0.000	0.027	**1.000**	**1.000**		**1.000**	**0.901**	0.143
SNP8	0.002	0.008	0.001	0.002	**0.166**	**0.166**	**0.166**		**0.960**	0.253
SNP9	0.001	0.001	0.000	0.001	**0.120**	**0.120**	**0.120**	**0.233**		0.148
SNP10	0.000	0.002	0.006	0.002	0.002	0.002	0.002	0.008	0.011	

For each pair of markers, the standardized D' is shown above the diagonal, and r^2 is shown below the diagonal

D' values of > 0.3 and r^2 values of > 0.1 are in boldface

*For SNP numbers, see Fig. 1.

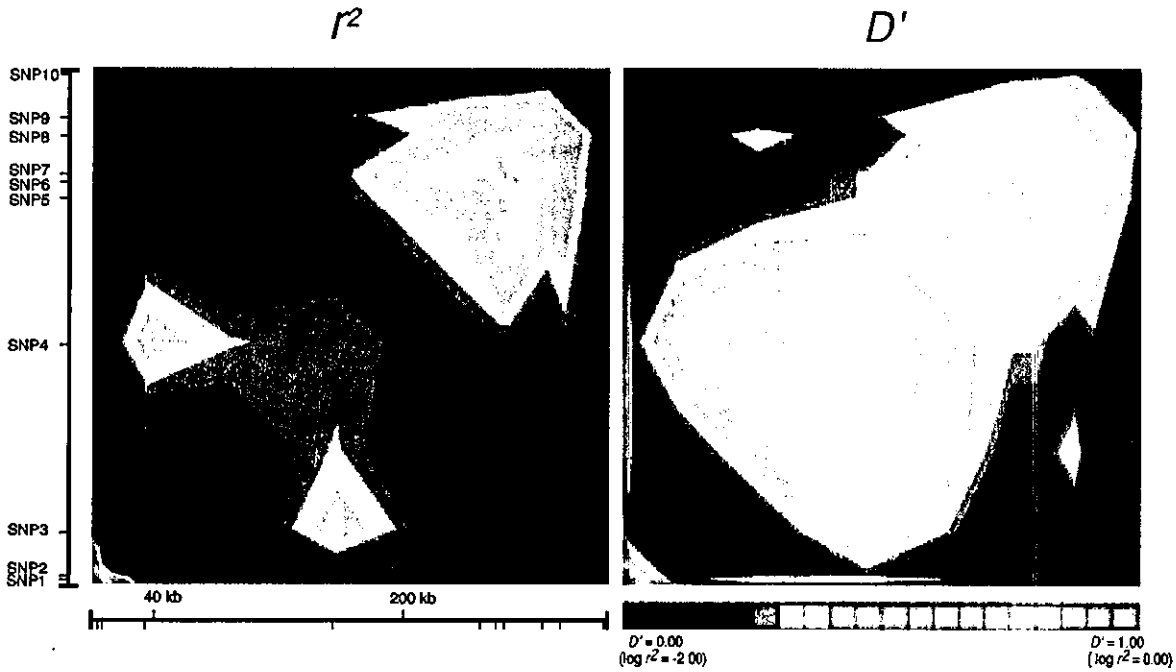

Fig. 2. LD map of the netrin G1 locus. GOLD plot of color-coded pair-wise disequilibrium statistics (r^2 in left and D' in right) is shown. Red and yellow indicate areas of strong LD. For SNP numbers, see Fig. 1.

Discussion

The mouse orthologue of netrin G1 gene was first cloned by Nakashiba *et al*[7] in 2000. They showed that netrin G1 transcripts were first detected at embryonic day 12 in mid and hindbrain regions, reaching peak levels at perinatal stages in various brain regions that include the olfactory bulb mitral cells, thalamus and deep cerebellar nuclei. Expression was primarily restricted to the central nervous system, with most prominent expression in the thalamus.[7] The thalamic neurons relay afferents to the cerebral cortex from various sensory systems, and thus the thalamus is deemed to modulate the motor response to sensory

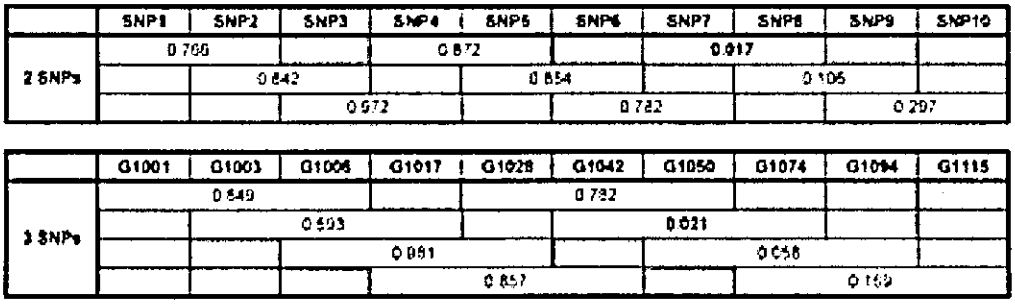

Fig. 3. Results of two-marker and three-marker haplotype analyses in schizophrenia. For two (three)-marker analysis, a sliding window of two (three) markers was tested, with one (two)-marker overlaps. The global *P* values shown, represent the overall significance when the observed versus expected frequencies of all of the haplotypes are considered together. The *P* values were calculated using the COCAPHASE program. For SNP numbers, see Fig. 1.

stimuli or to perform "sensory-motor gating".[12] Interestingly, netrin G1 knockout mice exhibited a reduced level of prepulse inhibition (PPI) (Itohara, personal communication). PPI is demonstrated by a reduction in response amplitude to a startle stimulus when this stimulus is immediately preceded by a weaker prestimulus.[13] This sensorimotor phenomenon occurs across-species and it has been investigated as a model to understand the pathophysiology of schizophrenia.[14] Schizophrenic patients often display profound impairments in PPI, raising the possibility that a deficit in the filtering or "gating" of sensory information may explain some of the fundamental symptoms observed in schizophrenia, including an overflow of sensory stimulation and disintegration of cognitive functions.[13] Therefore, netrin G1 is a potentially intriguing target for genetic studies in schizophrenia from both its role in physiological functions relevant to schizophrenia pathology, and its molecular involvement in neuronal circuit development.

Our case-control analysis revealed that the IVS8-1467C>T (SNP8) polymorphism of netrin G1 gene is nominally significantly associated with schizophrenia, with the IVS8-1467C allele overrepresented in schizophrenia (allelic $P = 0.020$, and > 0.05 after Bonferroni correction for multiple testing). Power calculations were performed based on an arbitrary assumption of relative risk and frequency of risk allele. When a relative risk of 2.0 was assumed, the present sample displayed 89% power to detect significant association ($\alpha < 0.05$, frequency of risk allele = 0.3, two-sided). With a relative risk of 1.5, our samples had 45% power to detect significant association ($\alpha < 0.05$, frequency of risk allele = 0.3, two-sided). For LD structure, Abecasis *et al*[15] suggested a *D'* value of greater than 0.33 as a useful measure of LD. Nakajima *et al*[16] proposed $r^2 > 0.1$ as a criterion for useful LD. According to their criteria, our LD analysis revealed that SNP8 was located in an LD block spanning from SNP5 to SNP9 with a gap between SNP5 and the neighboring SNP4. Haplotype analysis consistently showed that the haplotypes comprising SNP7-SNP8 and SNP6-SNP7-SNP8 in this LD block were associated with schizophrenia. These results suggest that the real disease-causing variant(s), if one exists, may reside in the 3' half region of the gene. We examined sequence variation in the exons and flanking introns using 40 schizophrenics and the primers shown in Table 1, but found no novel polymorphisms. In order to search for candidate functional variant(s), it is necessary to extend polymorphism screening to unscreened genomic regions.

The mouse genomic structure of netrin G1 gene is very similar to that of the human ortholog. Equally, we have detected various human netrin G1 transcripts generated by alternative splicing as seen in the mouse.[7] In humans, the splicing involves exons 6, 7, 8 and 9 which code for an unknown domain and two laminin repeat type domains (Meerabux *et al* in preparation). These exons are within the same haplotype block. Therefore it is tempting to speculate that dysregulation of transcript processing may have some role in schizophrenia susceptibility. However, the SNPs5, 6, 7, 8 and 9 are all embedded in introns and distant from branch points or splicing donor and acceptor sites, making them less likely to control the efficiency of alternative splicing. More thorough genomic and genetic analyses are needed to corroborate the contribution of netrin G1 gene to schizophrenia susceptibility and refine the predisposing allele(s). The close paralogue, netrin G2 gene, also identified from the mouse quite recently, also