Figure 6. Correlations between the TCI subscale score and the brain activity are plotted separately for each genotype group. Left, Harm avoidance (HA) score and right amygdaloid activity. AMG, Amygdala. Middle, Novelty-seeking (NS) score and activity in the right superior frontal gyrus (SFG; BA10). Right, Reward dependence (RD) score and medial prefrontal cortex (PFC; BA9) activity. Filled circles indicate subjects with C/C alleles, and open circles indicate subjects with C/T alleles. The regression lines (C/C group, solid line; C/T group, dotted line), correlation coefficients, and p values are also shown.
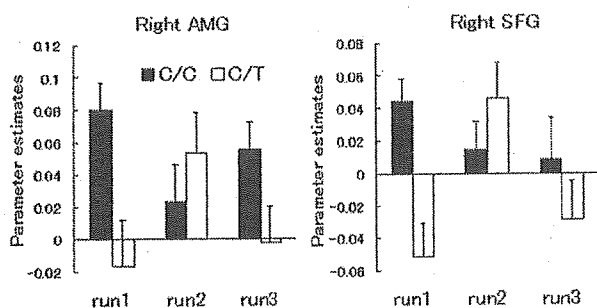

Figure 7. The mean and SE of the right amygdala (AMG; $x, y, z = 24, -4, -30$) and prefrontal [superior frontal gyrus (SFG); BA10; $x, y, z = 18, 52, 24$] responses to face stimuli in each group of subjects during the first (run1), second (run2), and third (run3) runs of the fMRI experiment are shown. ■, C/C group; □, C/T group.

typical temporal pattern of activation, whereas in the C/T group, the peak was observed in the second run. Reduced activation and the differential habituation pattern would be related to disturbance in stress hormone response through the 5-HT system (Van Praag, 2004).

Another significant finding was that the mPFC activation was greater in the C/C group than in the C/T group and positively correlated with the amygdaloid activation. Functional coupling of mPFC and the amygdala was significant during the first and the second runs in the subjects with C/C alleles. The mPFC plays a role in reward processing and goal-directed behavior in animals (Wise, 2000) and in humans (Rogers et al., 2004) and exerts significant control over the emotional–cognitive aspect of behavior (Vertes, 2004). Neuroanatomical studies in animals have shown that the mPFC has reciprocal connections with several nuclei of the amygdala (Ongur and Price, 2000), thalamus, and brainstem (Vertes, 2004) and relates with hormonal response to stress through the hypothalamic–pituitary–adrenal axis (Crane et al., 2003).

The present results have clinical implications in neuropsychiatry, because the C178T variant was more frequently found in bipolar affective patients than in a control group (Nielser et al., 2001). Differential BOLD signals and blood flow activation in the region of the amygdala, compared with normal subjects, have been observed in patients with mood disorders (Drevets, 2003) and anxiety disorders (Anand and Shekhar, 2003). An increased stress hormone level found in patients with depression and an anxiety disorder (Van Praag, 2004) is a biological marker of susceptibility to socio-psychological stress. Therefore, it is suggested

that the subjects with the T allele and an increased 5-HT$_3$ receptor expression (Nielser et al., 2001) may have an elevated hormonal level because the 5-HT$_3$ receptor is related to ACTH secretion in response to acute stress (Bhatnagar et al., 2004).

There are several caveats in the present study. First, the number of subjects is small and differed between the groups. In the future, a study with a larger number of subjects should be conducted. Second, the amygdala activity might have been limited because we used neutral faces, which elicit less activation in this region than faces expressing emotion, as stimuli. Third, the fact that there were no significant differences in the mean TCI subscale scores or BDI score between the genotypes may have obscured the functional relevance of the amygdala to personality and emotion. However, a recent study of HTR3A polymorphism and personality traits also showed no differences in the HA scores between the C/C and C/T groups (Melke et al., 2003). Neurophysiological responses as measured by fMRI would be more sensitive than questionnaires such as the BDI and TCI for detecting group differences. Neural activation in the amygdala should be treated as an endophenotype underlying both normal and pathological brain functions (Almasy and Blangero, 2001).

In conclusion, the present study, to the best of our knowledge, revealed for the first time the significant effect of HTR3A polymorphism on the amygdala and prefrontal activity as measured by fMRI. In addition, it showed that the difference in behavioral performance during a face recognition task was related to the genetic variation, indicating a close coupling of genes, behavior, and neurophysiological responses. The present findings may have clinical implications in predicting an individual's vulnerability to socio-psychological stressors before the onset of neuropsychiatric disorders. However, the causal relationship between genotype and brain activation is not fully elucidated in the present study. Therefore, additional investigation involving *in vitro* as well as *in vivo* experiments using animals, normal volunteers, and clinical populations are needed in the future.

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Association study of the frizzled-3 (FZD3) gene with schizophrenia and mood disorders

Short Communication

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Summary. Two research groups have recently reported a significant association between schizophrenia and genetic variants of Frizzled-3 (FZD3) gene. We examined a possible association in a Japanese sample of schizophrenia, bipolar disorder, unipolar depression and controls with four single nucleotide polymorphisms (SNPs), tested in previous reports. We failed to find significant association in the four SNPs or haplotype analysis. The FZD3 gene might not play a role in conferring susceptibility to major psychosis in our sample.

Introduction

Schizophrenia is a complex genetic disorder characterized by disturbances of cognition, emotion and social functioning. This disease is believed to involve genetic abnormalities in developmental/plasticity related processes during a critical period in neuronal growth (Weinberger et al., 2001). Wnt signal transduction cascades have been implicated in a variety of neurodevelopmental processes, e.g. segmentation, central nervous system patterning, and cell divisions (Wodarz and Nusse, 1998). Wnt proteins signal via cell surface transmembrane receptors, termed frizzleds, which display many properties

characteristic of members of the superfamily of G-protein-coupled receptors (Wang and Malbon, 2004). The frizzled-3 (FZD3) gene, a member of frizzles, is located on chromosome 8p21, repeatedly suggested as a positive linkage locus for schizophrenia (Lewis et al., 2003; McGuffin et al., 2003). The FDZ3 gene consists of 8 exons and 7 introns, spanning approximately 70 Kb (Kirikoshi et al., 2000). In accordance with this, two research groups have recently reported a significant association between schizophrenia and the FZD3 gene in Japanese and Chinese samples (Katsu et al., 2003; Yang et al., 2003). We tried to replicate these findings in an independent Asian sample. Furthermore, we also examined the possible association between the FZD3 gene with mood disorders, since schizophrenia and mood disorders might share the genetic vulnerability (Berrettini, 2003).

Methods and materials

Subjects

Subjects were 427 patients with schizophrenia (221 males and 206 females with mean age of 44.2 years [SD 14.5]), 91 with bipolar disorder (40 and 51; 53.6 years [SD 14.8]), and 396 with major depression (155 and 241; 53.4 years [SD 16.1]) and 473 healthy controls (228 and 245; 36.1 years [SD 12.5]). All the subjects were biologically unrelated Japanese. Consensus diagnosis was made for each patient by at least two trained psychiatrists according to the DSM-IV criteria. Controls were healthy volunteers who had no current or past contact to psychiatric services. After description of the study, written informed consent was obtained from every subject. The study protocol was approved by institutional ethical committees.

SNP genotyping

Venous blood was drawn from the subjects and genomic DNA was extracted from whole blood according to the standard procedures. We genotyped four SNPs (single nucleotide polymorphisms; dbSNP accession: rs960914 in intron3, rs2241802 in exon5: A435G, L145L, rs2323019 in intron5 and rs352203 in intron5) in the FZD3 gene, which were examined in the previous two studies (Katsu et al., 2003; Yang et al., 2003). Genotyping was performed with the TaqMan 5'-exonuclease allelic discrimination assay, described previously (Hashimoto et al., 2004a, b). Briefly, primers and probes for detection of the SNPs are: rs960914: forward primer 5'-CTTTTATAAAGAAATTTGAAACAT CAGAACATGGGA-3', reverse primer 5'-ACTTTTTCACTGCTTGGGAGTTATTCT-3', probe 1 5'-VIC-CTGAATGGCTGCTATC-MGB-3', and probe 2 5'-FAM-TCTGAATGGCTACTATC-MGB-3'; rs2241802:. forward primer 5'-ATGAGCCATATCCTCGACTTGTG-3', reverse primer 5'-GGACACCAAAAACCATAGTCTCTCT-3', probe 1 5'-VIC-TCCAGCTAAATTCAG-MGB-3', and probe 2 5'-FAM-CAGCCAAATTCAG-MGB-3'; rs2323019: forward primer 5'-GAAT TACTTTGTTTTTCTAGATTCTTGAATTGAAAGC-3', reverse primer 5'-CCAACCTGGTTAA TAATGGTCTTTTGG-3', probe 1 5'-VIC-TCATTTATTGTCAATGTTTTAA-MGB-3', and probe 2 5'-TCATTTATTGTCAATATTTTAA-MGB-3'; rs352203: forward primer 5'-CCTGAAAAAA TATTCTATATCTCTTGTTTTGCCA-3', reverse primer 5'-CAACCAGGACATAACAGTATTA CAGTTTCTAT-3', probe 1 5'-VIC-TCCTTCATGTCGTATTC-MGB-3', and probe 2 5'-FAM-TTTCCTTCATATCGTATTC-MGB-3'. PCR cycling conditions were: at 95°C for 10 minutes, 45 cycles of 92°C for 15 seconds and 60°C for 1 minute.

Statistical analysis

Statistical analysis of association studies was performed using SNPAlyse software (DYNACOM, Yokohama, Japan). The presence of Hardy-Weinberg equilibrium was examined using the $\chi\chi^2$ test for goodness of fit. Allele distributions between patients and controls were analyzed by the

Table 1. Allele distribution for SNPs in the FZD3 gene between major psychoses and controls

dbSNP ID	SNP	Controls	Schizophrenia		Bipolar		Unipolar	
		n = 473	n = 427	P value	n = 91	P value	n = 397	P value
rs960914	T/C	.398	.396	.91	.352	.23	.386	.61
rs2241802	G/A	.453	.458	.85	.451	.94	.455	.96
rs2323019	A/G	.407	.420	.57	.357	.21	.409	.91
rs352203	T/C	.397	.396	.97	.352	.24	.386	.64

χ^2 test for independence. The measures of linkage disequilibrium (LD), denoted as D', was calculated from the haplotype frequency using Expectation-Maximization algorithm. Case-control haplotype analysis was performed by the permutation method to obtain empirical significance (Good, 2000). The global p-values represent the overall significance using the χ^2 test when the observed versus expected frequencies of all the haplotypes are considered together. The individual haplotype was tested for association by grouping all others together and applying the χ^2 test with 1 df. P-values were calculated based on 10,000 replications. All p-values reported are two tailed. Statistical significance was defined at $p < 0.05$.

Results

The obtained allele frequencies for the patients and controls are shown in Table 1. The genotype distributions for all the diagnostic groups were in Hardy-Weinberg equilibrium (data not shown). There was no significant difference in genotype distributions or allele frequencies of the four SNPs in the FZD3 gene between the controls and any patient group, although previous studies reported positive associations between schizophrenia and several SNPs (Katsu et al., 2003; Yang et al., 2003). We computed the LD between the SNPs using D', which ranged between 0.8 and 1.0, indicating strong to intermediate LD between the markers. Adjacent combinations of up to four markers were tested, however, any haplotype combination was not significantly associated with any diagnostic group (all global p-values > 0.2).

Discussion

This study examined the possible association of the FZD gene with schizophrenia and mood disorders in our Japanese sample. We obtained no evidence for a significant association of the genetic variations of the FZD gene with any diagnostic group, suggesting that the examined polymorphisms play no major role in the pathogenesis of major psychoses in our sample. Our results are thus inconsistent with the results of the previous case-control study which reported a significantly higher frequency of the T allele of rs960914 in patients with schizophrenia than in controls (Katsu et al., 2003). The frequencies of the T allele were 0.62 and 0.51 in schizophrenics (n = 209) and controls (n = 200) in their Japanese sample, while the frequencies of the T allele were 0.60 and 0.60 in patients with schizophrenia (n = 427) and controls (n = 473) in ours. A highly significant association of the other three SNPs (rs2241802, rs2323019 and rs352203) and their three marker haplotypes with schizophrenia patients was reported in a family-based association study in a Chinese population

(Yang et al., 2003), while no evidence of such an association was obtained in our results (Chinese: global p-value < 0.000001, GAT haplotype p value < 0.000001; vs our results: global p-value $= 0.31$, GAT haplotype p-value $= 0.97$). Recently, the positive association between schizophrenia and the FZD3 gene has been reported in case-control study in a Chinese population (Zhang et al., 2004). This study presented that a new marker, rs880481, created the most positive results. Further analysis using this new marker should be examined in other ethnic populations.

A possible explanation for the discrepancy between the previous results and ours is a type II error in our sample. The odds ratio of the T allele of rs960914 was 1.54 in Japanese study (Katsu et al., 2003). However, power analysis revealed that our sample size could detect a significant association between the examined risk alleles (frequency of 0.4–0.6) and schizophrenia with a power of 90% when odds ratio was assumed to be 1.4 or more and the critical p-value was set at 0.05. There is only a small chance that a clinically meaningful difference would have been missed with the data. Secondly, it is possible that LD with other unknown polymorphisms, that is truly responsible for giving susceptibility to schizophrenia, may explain the discrepancy. Alternatively, the significant association observed by the previous two groups may have arisen by chance. The case-control association study is subject to the effect of population stratification, although the patients and controls were ethnically matched.

More recently, an extensive family-transmission and case-control analysis in a Japanese population with additional post-mortem mRNA expression data and family trio analysis in a British population yielded negative results for association between the FZD3 gene and schizophrenia (Ide et al., 2004; Wei and Hemmings, 2004), which is consistent with our results.

In conclusion, we obtained no evidence for an association between the FZD3 gene and schizophrenia or mood disorders, suggesting that this gene has no major role in conferring susceptibility to major psychoses in our sample.

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No association with the calcineurin A gamma subunit gene (PPP3CC) haplotype to Japanese schizophrenia

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Summary. Calcineurin, one of the serine/threonine protein phosphatase, comprises more than 1% of the total protein content in brain. This evidence points towards important roles of calcineurin in neural function. Miyakawa et al. reported that forebrain-specific calcineurin knockout mice showed the behavioral abnormalities that are often observed in schizophrenia patients. Based on this evidence, they suggested that calcineurin dysfunction could be involved in schizophrenia pathogenesis. Thereafter this report, Gerber et al. performed transmission disequilibrium test (TDT) studies and showed an evidence for a nominally significant over-transmission of a common haplotype of the human calcineurin A γ subunit gene (PPP3CC). We performed association analysis of PPP3CC in Japanese sample of 457 schizophrenia cases and 429 controls. To our regret, we could not confirm the association with Japanese schizophrenia to PPP3CC including core at-risk haplotype. Our result suggests that PPP3CC may not play a major role in Japanese schizophrenia.

Keywords: Single nucleotide polymorphism, calcineurin, haplotype.

Introduction

Phenotype of schizophrenia is characterized by positive and negative symptoms such as poor social function and cognitive dysfunction including attention deficit measured by pre-pulse inhibition test. Schizophrenia is also characterized by its fairly high heritability and abnormalities of glutamate (Moghaddam, 2003), γ-aminobutyric acid (GABA) (Wassef et al., 2003) and synaptic connectivity (Weinberger, 1999; Glantz and Lewis, 2001; Keshavan, 1999).

Recently, Miyakawa et al. reported that forebrain-specific calcineurin B subunit (CNB) knockout mice showed increased locomotor activity, decreased social interaction and impairments in pre-pulse inhibition similar to schizophrenic patients (Miyakawa et al., 2003). Calcineurin, one of the serine/threonine protein phosphatase, is highly enriched in neural tissue, as its concentration in brain is 10–20 times that found in other tissues and it comprises more than 1% of total protein content in the brain (Shibasaki et al., 2002; Klee et al., 1998; Wallace et al., 1980; Yakel, 1997; Guerini et al., 1990). Calcineurin also has been shown to have important roles in neural function. For example, activation of calcineurin inhibits the release of neurotransmitters, glutamate and GABA (Nichols et al., 1994; Greengard, 2001) and desensitization of the postsynaptic NMDA receptor (Tong, 1995; Lieberman, 1994). In addition, mice deficient in calcineurin-nuclear factor of activated T cells (NFAT) signaling had dramatic defects in axonal outgrowth (Graef et al., 2003). Considering the role of calcineurin in the glutamate and GABA system, in plasticity of neuron, and in social interaction and cognition, calcineurin would be a probable candidate gene for schizophrenia.

Thereafter Miyakawa's report, Gerber et al. performed transmission disequilibrium test (TDT) studies to examine the association of calcineurin-related candidate genes (calcineurin subunits, calcineurin binding proteins and proteins functionally coupled to calcineurin) with schizophrenia in 410 affected families collected from the United States (US) and South Africa (SA). They found an evidence for a nominally significant over-transmission of a common haplotype of the human calcineurin A γ subunit gene (PPP3CC) (Gerber et al., 2003) which is located within chromosome 8p21.3, a region that has been identified as schizophrenia susceptibility locus by linkage studies. Here, we performed a replication of their study with Japanese schizophrenia patients and controls.

Material and methods

Subjects

Subject consisted of 457 patients with schizophrenia (male: 242, female: 215) and 429 controls (male: 201, female: 228). All subjects were unrelated Japanese. All patients fulfilled the DSM-IV criteria for schizophrenia. The general characterization of these subjects and description of their psychiatric assessment were identical criteria published elsewhere (Suzuki et al., 2003). After description of the study, written informed consent was obtained from each subject. This study was approved by the Fujita Health University Ethics Committees.

Genotyping

Six single nucleotide polymorphisms (SNPs), five of which were the identical SNPs used in original study; SNP1 (CC-1a): rs1049437, SNP2 (CC-20): hCV1341797, SNP3 (CC-21): hCV1341817, SNP4 (CC-33): hCV3004214, SNP5 (CC-S3): rs246149 were genotyped. Another SNP (SNP6: rs7430 from dbSNP database (http://www.ncbi.nlm.nih.gov/SNP/) which is located in 3′ UTR was added as following reason. Denser SNP setting would be required especially in latter half of PPP3CC, because original positive results showed that SNPs located in latter half of PPP3CC were associated with schizophrenia.

Genomic DNA was extracted from peripheral blood of all subjects. For rapid genotyping of SNPs, polymerase chain reaction (PCR) restriction fragment length polymorphism (RFLP) assays and TaqMan assays were developed.

Table 1. Primers and restriction enzymes used for genotyping

SNP	PCR Primer sequences	Product size (bp)	Annealing temperature	Restriction enzymes	Variants	Location
CC-20	5′-GGGCACAGAAAGATATACTGG-3′ 5′-GTAGAAAGTCCCACAAACTGG-3′	278	61	Xba I	T/C	Intron 1
CC-21	5′-GAAGAAGCTTCTGGCATCTGG-3′ 5′-TAGTCCCATGATCCACAGGTG-3′	312	56	Hinf I	G/A	Intron 1
CC-33	5′-GACAGAGTGAGACCCTGTCC-3′ 5′-TGACCTGCATTGACCTACATC-3′	197	56	Hpy188I	C/T	Intron 4
CC-S3	5′-GATGGAACAATAGGTCCTGG-3′ 5′-CATGCAGAGCAACACACATG-3′	266	61	Pml I	A/G	Intron 4
rs 7430	5′-AAGGGAAGAAAGCCCATTCA-3′ 5′-AAGGGAAAAACAAAACAGATGC-3′	238	56	BstNI	C/G	Untranslated

Five pairs of primers were used for typing SNP2, SNP3, SNP4, SNP5 and SNP6 for PCR-RFLP, as described in Table 1.

PCR amplification was carried out as follows. The reaction mixture was in a $6\,\mu l$ volume containing a 10 ng sample DNA, 10 pmol of each primer, 2.5 mM $MgCl_2$, each dNTP at 0.2 mM and 0.3 U of AmpliTaq Gold^{TM} (Applied Biosystems Japan Ltd.). An initial denaturation step at 95°C 9 min was followed by 40 cycles of denaturation at 95°C for 15 sec, primer annealing at each suitable temperature for 20 sec, primer extension at 72°C for 30 sec and a final extension reaction was performed at 72°C for 7 min. The PCR products were digested with each appropriate restriction enzyme (Table 1). DNA fragments were resolved by electrophoresis in a 6% acrylamide gel stained with ethidium bromide.

Concerning SNP1, the primer that was used in original study for typing SNP1 was so long that we could not type it exactly. Then we typed SNP1 with TaqMan assays (Applied Biosystems Japan Ltd.).

Statistical analysis

The presence of Hardy-Weinberg equilibrium (HWE) was examined by exact test. D' and r^2 were calculated to evaluate pairwise linkage disequilibrium (LD). Genotype, allele and haplotype distributions between cases and controls were analyzed by COCAPHASE 2.403 (Dudbridge, 2003).

In conventional haplotypic analyses, we estimated the haplotype frequencies by expectation–maximization (EM) algorithm. We set cutoff line of haplotype frequencies less than 5% as original study defined.

An effect size was estimated by power, which was calculated using a statistical program prepared by Ohashi (Ohashi et al., 2001). The significance level for all statistical tests was 0.05.

Results

Genotype distributions in schizophrenic patients and controls are shown in Table 2. In each SNP, the genotype distributions for the two groups were in Hardy-Weinberg equilibrium. No significant differences in genotype of all SNP were observed between Japanese schizophrenic patients and controls.

Pairwise disequilibrium values of Japanese schizophrenia sample were shown in Table 3. Almost all pairwise LD and r^2 results showed the strong LD, however, CC-20 is not in strong LD with CC-33 and CC-S3 in combined

Table 2. Genotypic analysis of PPP3CC

SNP ID	Japanese sample (Frequencies in %)						
	M/M		M/m		m/m		P-value
	Patients	Controls	Patients	Controls	Patients	Controls	
CC-1a	131 (28.7)	123 (26.9)	214 (46.8)	204 (47.6)	112 (24.5)	102 (23.8)	0.88
CC-20	323 (70.7)	312 (72.7)	123 (26.9)	121 (28.2)	11 (2.4)	16 (3.7)	0.27
CC-21	222 (48.6)	220 (51.3)	195 (42.7)	179 (41.7)	40 (8.8)	30 (7.0)	0.29
CC-33	156 (34.1)	144 (33.6)	211 (46.2)	210 (49.0)	90 (19.7)	75 (17.5)	0.73
CC-S3	160 (35.0)	147 (34.3)	218 (47.7)	211 (49.2)	79 (17.3)	71 (16.6)	0.99
rs 7430	146 (31.9)	135 (31.5)	219 (47.9)	222 (51.7)	92 (20.1)	72 (16.8)	0.51

M major allele; m minor allele

Table 3. Pairwise disequilibrium values for PPP3CC

	Japanese sample						US sample*				Distances (bp)
	CC-1a	CC-20	CC-21	CC-33	CC-S3	rs 7430	CC-1a	CC-20	CC-21	CC-33	
CC-1a		0.08	0.36	0.58	0.55	0.25					
CC-20	0.58		0.06	0.0004	0.0003	0.08	0.72				6843
CC-21	0.89	0.87		0.46	0.49	0.38	0.98	0.78			15305
CC-33	0.85	0.04	0.91		0.84	0.34	0.89	0.39	0.67		38764
CC-S3	0.85	0.05	0.92	0.94		0.39	0.97	0.51	0.9	0.91	1683
rs 7430	0.55	0.72	0.84	0.6	0.65						37162

For each pair of markers, the standardized D' is shown below the diagonal, and r^2 is shown above the diagonal.
* Gerber et al. (2003)

Table 4. Global haplotype association analysis of PPP3CC in Japanese Case-Control Samples

SNP ID	Global haplotype analysis (p-value[a])					
	1SNP*	2SNP	3SNP	4SNP	5SNP	6SNP
CC-1a	0.87					
		0.72				
CC-20	0.26		0.75			
		0.39		0.97		
CC-21	0.29		0.74		0.9	
		0.79		0.77		0.87
CC-33	0.72		0.75		0.63	
		0.92		0.68		
CC-S3	0.99		0.67			
		0.66				
rs 7430	0.51					

[a]p-value was calculated by COCAPHASE. * allele wise associate

Table 5. Individual haplotype analysis (5SNPs CC-1a ~ CC-S3)

5SNPs haplotype (CC-1a ~ CC-S3)	Japanese sample			US sample[***]		South Africa (SA) sample[***]		US + SA[***]
	Frequencies (%)		P-value[a]	Frequencies (%) (210 triads)	P-value[b]	Frequencies (%) (200 triads)	P-value[b]	P-value[b] (410 triads)
	SCZ (n = 457)	CON (n = 429)						
CTGCA	26.4	26.4	0.98	38	0.002[*]	43	0.07	0.0012[**]
CTACA	5.7	5.8	0.88	8	0.48	9	0.32	
CTATG	1.2	0.9	0.28	11	0.15	8	0.32	
ATATG	43.9	45.8	0.47	25	0.7	26	0.35	
CCACA	4.6	5.7	0.32					
CCATG	6.4	7.2	0.49					

SCZ schizophrenic. *CON* control. [a] Calculated by COCAPHASE. [b] Calculated by TRANSMITED. [*] In TDT results of the US samples showed a risk haplotype. [**] In TDT results of the US + SA samples also showed a risk haplotype. [***] Gerber et al. (2003)

sample of schizophrenics and controls. Also same trends were obtained from separated samples of schizophrenics and controls (data not shown).

Results of allele and haplotypic analysis are shown in Tables 4, 5 and 6. No significant differences in allele frequencies for all SNP were observed between Japanese schizophrenic patients and controls, including CC-21 (P = 0.75) and CC-S3 (P = 0.44) that were observed significant over-transmission in US and SA samples (P = 0.038 and 0.041, respectively).

For the conventional haplotypic analysis, like original study, we evaluated associations by two forms, global haplotypic analysis, and individual haplotypic analysis. In both analyses we constructed from two to six SNPs haplotypes from adjacent SNPs in sliding-window fashion (Table 4). In global haplotypic analysis, none of the five SNPs showed association with

Table 6. Individual haplotype analysis (6SNPs CC-1a/rs 7430)

6SNPs Haplotypes (CC-1a ~ rs 7430)	Japanese sample		
	Frequencies (%)		P-value[a]
	SCH (n = 457)	CON (n = 429)	
ATATGC	35.5	38.3	0.24
CTGCAG	24.8	25.4	0.79
ATATGG	8.5	7.5	0.39
CCATGC	5.5	6.4	0.48
CTACAG	5.6	5.7	0.94

[a] Calculated by COCAPHASE

schizophrenia. In individual haplotypic analysis of 5 SNPs for the confirmation of the contribution of individual haplotypes to global haplotypic analysis, we could not find association of major haplotypes including at-risk haplotypes (Table 5). Concerning individual haplotypic analysis of 6 SNPs (Table 6), we could not reveal association of major haplotypes to schizophrenia either.

Discussion

We have performed the first replication of association study of PPP3CC using relatively large Japanese case-control samples. For denser SNP setting, we added another SNP that is located in 3' UTR. Although the original study showed significant associations with 2 SNPs (CC-21, CC-S3) in PPP3CC to schizophrenia, in our Japanese sample, all SNPs in PPP3CC did not show significant association with schizophrenia (Tables 2 and 4). In haplotypic analysis, we could not find significant association of major haplotypes including at-risk haplotype, either (Tables 4, 5 and 6). Our results did not confirm the previous association of PPP3CC.

This discrepancy between our study and original study might be derived from differences of the population. Japanese samples differ from US and SA samples in LD patterns (Table 3). Also, haplotypes were present in different frequencies in Japanese sample and US/SA sample (Table 5).

In addition, the magnitude of susceptibility loci from linkage analyses may be different among populations. Genome-wide linkage studies, primarily of European-Caucasian, African-American and Palauan populations, have been performed and some studies have suggested that 8p.21-22 (Pulver et al., 1995; Kendler et al., 1996; Blouin et al., 1998; Gurling et al., 2001; Straub et al., 2002), where PPP3CC is located on, is associated with susceptibility to schizophrenia. However, in genome-wide scan for linkage with Japanese schizophrenia, 8p.21-22 did not fulfill the criteria for significant or suggestive evidence for linkage (JSSLG, 2003) (Yamada et al., 2004).

To verify that our negative results might not be due to type II error, traditional power calculation based on genotype relative risk (GRR) was performed. The power was quite high and showed more than 80% power for susceptibility gene when GRR set 1.31–1.45 (Ohashi et al., 2001).

In conclusion, our results suggested that PPP3CC might not play a major role in pathophysiology of Japanese schizophrenia. However, as calcineurin is still a probable candidate gene for schizophrenia, further association analyses is required to be carried out considering locus heterogeneity among calcineurin and calcineurin-interacting molecules.

Acknowledgements

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No association of serotonin transporter gene (SLC6A4) with schizophrenia and bipolar disorder in Japanese patients: association analysis based on linkage disequilibrium

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Summary. Serotonin transporter gene (SLC6A4) is one of the most promising candidate genes for psychiatric disorders such as schizophrenia (SCZ) and bipolar disorder (BP). Two functional polymorphisms, 5HTTLPR and 5HTTVNTR, have been a focus for genetic association analyses; however, no conclusive results have been obtained. We conducted, 1) a mutation search of SLC6A4, 2) LD mapping to select 'tagging' markers (10 SNPs and 5HTTVNTR, while 5HTTLPR was treated as an independent marker because of its allelic form), and 3) association analysis of these 'tagging' markers and independent markers (5HTTLPR and Asn605Lys) with SCZ and BP in Japanese patients. In this mutation search, a nonsynonymous SNP, Asn605Lys, was detected. No associations of 'tagging' markers and independent markers with such conditions were found. These results indicate that SLC6A4 might not play a major role in SCZ and BP in Japanese patients, a finding that agrees with both the common disease-common variant hypothesis and common disease-rare variant hypothesis.

Introduction

Serotonin transporter gene (5HTT, SLC6A4) is one of the most promising candidate genes for psychiatric disorders, such as schizophrenia (SCZ) and bipolar disorder (BP), based on its import roles in serotonin transmission and the pharmacological mechanism of various antidepressants (OMIM: *182138). Several genetic association analyses of SLC6A4 have been performed in different population samples. These association analyses have focused on two functional polymorphisms, 5HTTLPR (5HTT "Long/Short", a 44-bp insertion/deletion, Polymorphism) and 5HTTVNTR (5HTT a 17-base-pair (bp) variable-number tandem-repeat (VNTR) in intron 2). For SCZ, results from several association studies have been inconsistent (e.g. Serretti et al., 2002; Tsai et al., 2002). On the contrary, for BP, two recent meta-analyses of 5HTTLPR and/or 5HTTVNTR showed significant

association with affective disorder (Anguelova et al., 2003; Lasky-Su et al., 2005). However, while meta-analysis is unquestionably a powerful tool for genetic association analysis, it has several limitations based on publication bias, ethnic diversity and other factors.

A popular hypothesis about allelic architecture proposes that most genetic risk for common, complex diseases including SCZ and BP is due to disease loci where there is one common variant (Chakravarti, 1999). If true, this common disease-common variant (CD-CV) hypothesis implies that linkage disequilibrium (LD) mapping is an important concept in order to narrow the predisposing polymorphisms for complex diseases in association analysis, rather than focusing on specific polymorphisms. To represent LD properties, single nucleotide polymorphisms (SNPs) are often used as haplotype tagging markers, because SNPs are abundant and amenable to genotyping. Applying these concepts, LD-based analysis could provide different insights from previous analyses using only independent markers such as 5HTTLPR and 5HTTVNTR.

To date, only one association study based on LD, which examined the association of SLC6A4 with BP (not with SCZ), has been reported (Sun et al., 2004).

Alternatively, a common disease-rare variant (CD-RV) hypothesis (Pritchard and Cox, 2002) was proposed recently, stemming from criticisms of the CD-CV hypothesis from the viewpoints of allelic heterogeneity and disease heterogeneity. According to the CD-RV hypothesis, it is important to search for rare and functional variants as possible disease-related variants.

In the present study, we conducted 1) a mutation search of all exons and possible promoter regions (2064 bp around 5HTTLPR), 2) LD mapping (10SNPs and 5HTTVNTR) and selection of 'tagging' markers, and 3) a case-control association analysis of SCZ and BP using these 'tagging' markers, 5HTTLPR

and a rare variant detected in our mutation search.

Material and methods

Subjects

The subjects for the mutation search were 37 and 27 patients with SCZ and bipolar I disorder, respectively. LD mapping was performed in 96 controls. In the following association analysis, 287 patients with SCZ (148 male and 139 female; mean age ± standard deviation (SD) 42.3 ± 14.7 years), 109 patients with BP (51 male and 58 female; 61 patients with Bipolar I disorder and 48 patients with bipolar II disorder; mean age ± SD 48.3 ± 13.2 years) and 288 controls (150 male and 138 female; mean age ± SD 33.6 ± 12.9 years) were genotyped. They were diagnosed according to DSM-IV criteria with the consensus of at least two experienced psychiatrists on the basis of unstructured interviews and review of the medical records. All healthy controls were also psychiatrically screened based on unstructured interviews. All subjects were unrelated each other and ethnically Japanese. More detailed characterization of these subjects and description of their psychiatric assessment are identical to those published elsewhere (Suzuki et al., 2003). After explanation of the study, written informed consent was obtained from each subject. This study was approved by the Ethics Committee at Fujita Health University and Nagoya University Graduate School of Medicine.

SNP identification

Genomic DNA was extracted from peripheral blood of all subjects. For mutation search, primer pairs were designed using information from the GenBank sequence (accession number: NT-01799.13) and 22 amplified regions, which covered all of the coding regions and introns including the branch sites and 2064 bp around 5HTTLPR. We also developed a denaturing high-performance liquid chromatography (dHPLC) analysis. A more detailed description can be seen in a previous paper (Suzuki et al., 2003). Sequences of primer pairs are available on request.

LD mapping and SNP genotyping

For LD mapping, we included SNPs from databases (dbSNP; http://www.ncbi.nlm.nih.gov/SNP/ and Celera Discovery Systems; http://www.celeradiscoverysystem.com/) and other papers (Battersby et al., 1999), so that the SNPs were evenly distributed (Table 1). First we determined 'LD blocks'

Table 1. SNPs in LD mapping

ID		Distance to next marker	Method of genotyping	MAF of controls[1] (%)
Private IDs	Database IDs			
M1	rs1050565	–	TaqMan	15.1
–	5HTTLPR[2]	11563	PCR	–
2	rs2020934	3053	PCR-RFLP	15.1
3	rs2066713	9795	direct sequencing	6.25
4	rs2020936	851	PCR-RFLP	13.0
5	rs2020937	57	direct sequencing	6.78
6	rs2020938	7	direct sequencing	14.3
7	rs2020939	18	direct sequencing	14.6
8	5HTTVNTR	2104	PCR	5.73
9	rs140701	10096	direct sequencing	16.7
10	rs3794808	6739	PCR-RFLP	15.1
–	Asn605Lys (rs6352)[2]	1600	PCR-RFLP	–
11	rs3813034	5389	PCR-RFLP	16.1

[1]MAF = minor allele frequency of 96 controls used in LD mapping. [2]These variants were not included in LD mapping (see text)

with reasonable criteria based on 95% confidential bounds on D' values using the Haploview ver. 2.05 software (Barrett et al., 2005). Next, 'tagging' markers were selected within each 'LD block' for 90% haplotype coverage using SNPtagger software (Ke and Cardon, 2003). In this case, we preferred to select possible markers with functional relevance rather than just intronic markers.

Genotyping of 5HTTLPR, M8 (5HTTVNTR), and M11 (rs3813034, 3' UTR G > T) was as described in other papers (Battersby et al., 1999; Lesch et al., 1996; Ogilvie et al., 1996). For rapid genotyping of the other markers used in our LD mapping, we used TaqMan assays, restriction fragment length polymorphism (RFLP) assays, primer extension methods using dHPLC, and a direct sequencing method (Table 1). TaqMan probes and Universal PCR Master Mix were obtained from Applied Biosystems. A 5-µl total reaction volume was used and, after PCR, the allelic specific fluorescence was measured on ABI PRISM 7900 Sequence Detector Systems (Applied Biosystems). RFLP assays and primer extension methods were described in greater detail previously (Suzuki et al., 2003). Detailed information including primer sequences is also available on request.

Statistical analysis

Genotype deviation from the Hardy-Weinberg equilibrium (HWE) was evaluated by χ^2 test (SAS/genetics, release 8.2, SAS Institute Japan Inc, Japan).

Marker-trait association was evaluated allele/genotype-wise with conventional χ^2 test or Fisher's exact test (SPSS 10.0J, SPSS Japan Inc, Japan) and haplotype-wise with the program COCAPHASE 2.403 (Dudbridge, 2003). The COCAPHASE program performs log-likelihood ratio tests under a log-linear model for global P-value. To estimate haplotype frequencies of 'tagging' markers, the expectation-maximization (EM) algorithm was used. Rare haplotypes found in less than 3% of both cases and controls were excluded from association analyses to provide greater sensitivity and accuracy when the effect is seen in common haplotypes. And for haplotype-wise analyses, we calculated global P-values in sliding-window fashion.

Power calculation was performed using a statistical program prepared by Ohashi et al. (2001). We estimated the power for our sample size under a multiplicative model of inheritance assuming a population susceptibility allele frequency of each value in our screened control samples (type I error rate = 0.05).

The significance level for all statistical tests was 0.05.

Results

In our mutation search, only a nonsynonymous single nucleotide polymorphism (SNP), Asn605Lys (rs6352), was detected in one SCZ; no mutation was found in BP. Due to its low minor allele frequency (MAF), we did not
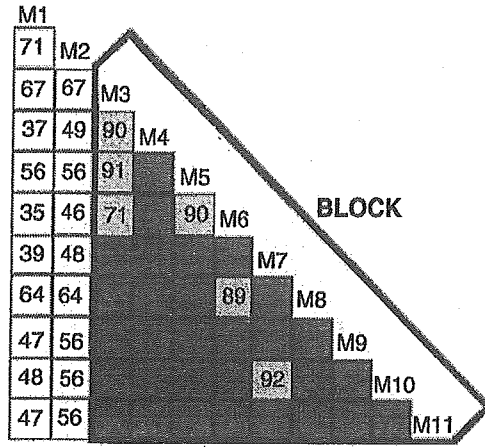
Fig. 1. Linkage disequilibrium mapping. Numbers in box represents D′ values after decimal point. D′ values of 1.0 are not shown. Color schemes represent the evidence of LD or recombination (strong evidence of LD: dark gray, uninformative: light gray, strong evidence of recombination: white). The other information is described at Haploview's website

include this SNP in the following LD mapping. Next, LD mapping of controls showed one LD block covering all of the coding exons (Fig. 1), and M11 and M8 (5HTTVNTR) were selected as 'tagging' markers from this block. Consequently, four markers (M1, M2, M8 and M11) were selected as those of SLC6A4 (Table 1). In this case-control association analysis, we treated 5HTTLPR and Asn605Lys as independent markers, because 5HTTLPR is a complex multi-allelic variant (Nakamura et al., 2000) and MAF of Asn605Lys was too low, as described above.

We first evaluated the deviations from HWE for all markers, and found that genotype frequencies were consistent with HWE (for M8, 5HTTVNTR, 9 repeat allele was not found in either our case and control samples). No associations of 'tagging' markers and 5HTTLPR with SCZ and BP were found (Table 2). However, because Asn605Lys

Table 2. Allele/genotype-wise association analysis of 'tagging' markers with SCZ and BP

Phenotype[1]	ID	Number	Genotype[2]			P-values		Power calculation[3]
			M/M	M/m	m/m	Genotype	Allele	GRR
SCZ	M1	287	196	79	12	0.139	0.343	1.47
	2	287	208	68	11	0.161	0.594	1.51
	8[4]	287	237	49	1	0.350	0.583	1.78
	11	287	196	83	8	0.781	0.557	1.49
	5HTTLPR	287	189	82	16	0.214	0.441	1.46
	Asn605Lys	381	353	28	2	0.911	0.541	1.95
BP	M1	109	75	30	4	0.377	0.389	1.67
	2	109	76	30	3	0.867	0.912	1.71
	8[4]	109	93	15	1	0.993	0.930	1.99
	11	109	70	33	6	0.420	0.509	1.68
	5HTTLPR	109	73	30	6	0.339	0.452	1.65
	Asn605Lys	109	100	8	1	0.515	0.489	2.55
Controls	M1	288	180	100	8			
	2	288	197	85	6			
	8[4]	288	245	40	3			
	11	288	189	91	8			
	5HTTLPR	288	175	101	12			
	Asn605Lys	351	327	23	1			

[1]SCZ schizophrenia, BP bipolar disorder. [2]M major allele, m minor allele. [3]GRR genotype relative risk to obtain 80% power. [4]5HTTVNTR: M (major allele) = 12 repeat, m (minor allele) = 10 repeat

Table 3. Haplotype-wise association analyses

Phenotype[1]	ID	Global P-values in sliding-window fasion		
		2SNP	3SNPs	4SNPs
SCZ	M1	0.651	0.376	0.715
	2	0.701	0.545	
	8	0.151		
	11			
BP	M1	0.675	0.847	0.927
	2	0.910	0.903	
	8	0.878		
	11			

[1]SCZ schizophrenia, BP bipolar disorder

showed weak association with SCZ (P = 0.0338), we expanded the search in a further 96 schizophrenics and 64 control samples (total schizophrenia = 383, control = 351) for conclusive results. In this additional analysis, we could not confirm the positive association of Asn605Lys with SCZ (P = 0.541: Table 2). In addition, we performed a haplotype-wise association analysis of sliding-window fashion using these four 'tagging' markers. Again, we found no association with SCZ and BP (Table 3).

The Power calculations are shown in Table 2. We obtained more than 80% power to detect association when we set the genotype relative risk (GRR) at each value as shown in Table 2.

Discussion

In this study, no associations of SLC6A4 with SCZ and BP in Japanese patients were found in accordance with either the common disease-common variant (CD-CV) hypothesis or common disease-rare variant (CD-RV) hypothesis (Pritchard and Cox, 2002).

The association analysis strategy we have adopted is reasonable for genetic association study of SLC6A4 for the following reasons. 1) Mutation search with relatively high power can detect SNPs that are rare but have functional effect (Collins and Schwartz, 2002), and avoid overlooking associations in accordance with the CD-RV hypothesis. In fact, one of us reported that a rare but functional SNP of SLC6A4 (Ile425Val) was associated with severe familial obsessive-compulsive disorder, and suggested that such a rare variant of SLC6A4 is also important in terms of susceptibility genes for complex disorders (Ozaki et al., 2003). We included the mutation scan of this SNP using 500 schizophrenics, however, no mutation was found in our samples. And another nonsynonymous SNP from database, Ala56Gly (rs6355), was also searched for 96 schizophrenics, again no mutation was found (data not shown). 2) Our association analyses using 'tagging' markers were more sensitive and useful than those using randomly selected markers. Moreover, our 'tagging' markers could reflect the haplotype background of SLC6A4 in the Japanese population, especially in coding regions. And it is reasonable to treat 5HTTLPR as an independent marker, because 5HTTLPR is a multi-allelic variant and the arbitrary division ('L' or 'S' allele) cannot estimate the exact LD structure of SLC6A4.

A recent association study based on LD of Taiwanese BP found an association with the fifth commonest haplotype (Sun et al., 2004). However, our haplotypic analysis did not show significance either in global haplotypic analysis or even in individual haplotypic analyses (data not shown). This discrepancy between our results and Sun's might be due to a difference in the study populations.

We also included an explorative analysis of 5HTTLPR and 5HTTVNTR, because a recent expression study reported that allelic combination of 5HTTLPR and 5HTTVNTR showed weak but significant differences in serotonin transporter mRNA levels (Hranilovic et al., 2004). They reported that genotypes were separated into three groups, no 'low-expressing' at either of the loci (L/L, 12/12), 'low-expressing' at one locus (L/L, '10' allele and