eral studies obtained evidence supporting this association [6–9], although contradictive negative results have also been reported [10–12]. Interestingly, individuals with schizophrenia who carried the A3 allele had smaller hippocampi than those who did not [13].

With respect to bipolar disorder, there is evidence for a significant association of the illness with a single nucleotide polymorphism (Val66Met) and a dinucleotide repeat polymorphism of the brain-derived neurotrophic factor (BDNF) gene [14, 15]. BDNF also belongs to the neurotrophin family and its primary structure is highly homologous to that of NTF3. Thus, NTF3 is an attractive candidate gene that may give susceptibility to bipolar disorder as well as to schizophrenia, which accords with substantial evidence that these disorders share familial risk characteristics [16].

Recently, micro- and minisatellite polymorphisms, even located in intron, have been shown to play a role in the expression of many genes [17]. In this study, we tested the hypotheses that the dinucleotide repeat polymorphism of the NTF3 gene may give susceptibility to bipolar disorder and that the polymorphism may have functional effects on transcriptional activity in an allele-dependent manner.

Materials and Methods

Subjects

To examine whether the dinucleotide repeat polymorphism of the NTF3 gene is associated with bipolar disorder, we genotyped 88 patients [38 men and 50 women; mean age 53 years, standard deviation (SD) 15] with bipolar disorder and 98 controls (44 men and 54 women; 51 years, SD 10). The patients were under treatment at Showa University Hospital, Tokyo, Japan. Consensus diagnosis was made for each patient by at least 2 psychiatrists according to the Diagnostic and Statistical Manual of Mental Disorders (ed 4, DSM-IV) criteria [18] based on unstructured interviews and all the available medical records. Forty-six patients (52%) were diagnosed as having bipolar I and the remaining 42 bipolar II disorder. The controls were healthy volunteers recruited from the hospital staff who had no current or past contact to psychiatric services for psychiatric problems. All the participants were unrelated Japanese who resided in Tokyo metropolitan area. Written informed consent was obtained from every subject after detailed description of the study. The study protocol was approved by institutional ethical committees.

Genotyping

Venous blood was drawn from the subjects and genomic DNA was extracted from whole blood according to standard procedures. The dinucleotide repeat polymorphism of the NTF3 gene was genotyped by polymerase chain reaction (PCR) amplification followed by determination of PCR fragment sizes by using an autosequencer (CEQ8000, Beckman Coulter) and a CEQ Fragment Analysis System (Beckman Coulter). Primer sequences for the PCR were 5′-(dye D4) GTTTGCGCTGGGATCTGCTCA-3′ (forward) and 5′-CCCCCA-CCCTTTCCAATCCA-3′ (reverse). Thermal cycling for the PCR was an initial denaturing stage at 95 °C for 9 min, 34 cycles of 95 °C for 45 s, 62 °C for 45 s, and 72 °C for 30 s, followed by the final extension stage at 72 °C for 10 min. Genotype data were read blind to the case-control status.

Luciferase Reporter Gene Assay

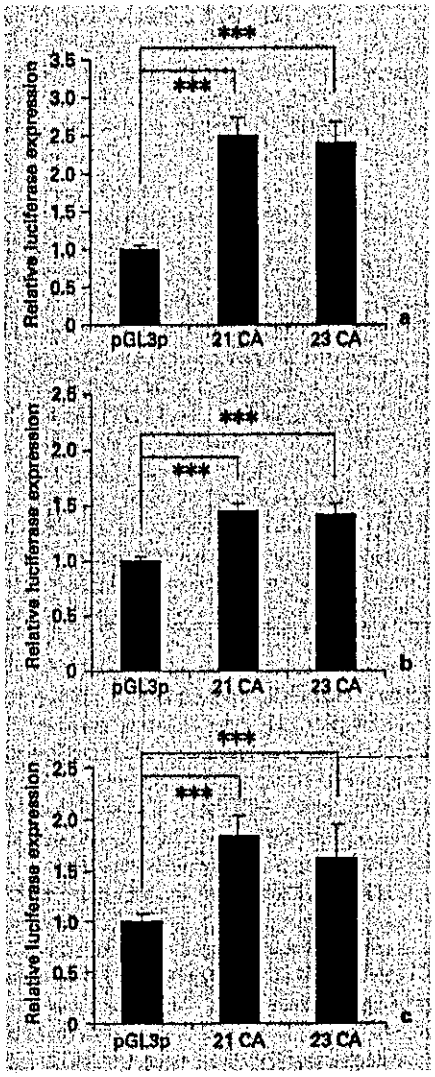
The CA repeat region in the first intron of the NT3 gene was amplified by PCR. The oligonucleotide primers were designed to incorporate Mlu I (forward) and Bgl II (reverse) restriction sites to the primers of the previous report [4], and the PCR product was inserted into the polylinker site upstream of the SV40 promoter in the pGL3-promoter vector (Promega). The primer sequences were 5′-GCACGCGTGGCTTGTGTCTTCCCCAAAGTT-3′ (forward; restriction site is underlined) and 5′-GCAGATCTAGGGGAG-GAGGTGGAGAA-3′ (reverse). According to allele frequency (see 'Results'), the major alleles were the A3 allele of $(CA)_{23}$ and A5 of $(CA)_{21}$ (153 and 149 bp, respectively), and thus these two alleles were inserted into the vector for comparison. The insert sequence was confirmed with the autosequencer (CEQ8000, Beckman Coulter) in both directions using primers supplied by the manufacturer (Promega; RV primer 3 and GL primer 2).

Human neuroblastoma IMR-32 cells and HeLa cells were cultured in MEM (Sigma) containing 10% fetal bovine serum (GIBCO). Human glioma Hs683 cells were cultured in DMEM (Sigma) containing 10% fetal bovine serum. Hs683 and IMR-32 cell lines were chosen according to previous reports on promoter assays of the NTF3 gene [19, 20]. Cells in 24-well plates were cotransfectioned in triplicate with 800 ng of pGL3-promoter firefly luciferase reporter vectors that included two major alleles of the $(CA)_n$ repeats (n = 21 and 23) and 25 ng of renilla luciferase expression vector (phRL-TK vector; Promega) as an internal control using Lipofectamine 2000 Regent (Invitogen). Empty pGL3-promoter vector was transfected simultaneously in the experiments. Experiments were repeated three times in all cells.

At 24 h after transfection, cells were washed with phosphate-buffered saline and then harvested with luciferase lysis buffer (Promega). Luciferase reporter assay was performed using Dual-Luciferase Reporter Assay System (Promega) and a luminometer (Lumat LB 9507, Berthold). Firefly and renilla luciferase activities were quantified sequentially as relative light units (RLU) by addition of their respective substrates according to the protocol of the supplier. The ratio of a firefly RLU to renilla RLU of each sample was automatically computed. Then the activity of each construct was expressed as the relative value compared to that of empty pGL3-promoter vector (relative luciferase expression, RLE), and these relative values were used for statistical analysis.

Statistical Analysis

For the association study, the presence of Hardy-Weinberg equilibrium was examined by using the χ^2 test for goodness of fit. Allele frequency was compared between patients and controls and the significance was assessed with a Monte Carlo approach using the CLUMP software with 10,000 simulations [21]. For luciferase reporter gene assay, analysis of variance (ANOVA) was used for comparison of RLE between alleles inserted into vectors. All p values reported are two-tailed.



Results

Association Analysis

The genotype distributions for the patients and controls did not significantly deviate from Hardy-Weinberg equilibrium (data not shown). Allele frequencies for the dinucleotide repeat polymorphism in the NTF3 gene among the patients and controls are shown in table 1. The observed allele frequencies among the two groups were quite similar. Based on the Monte Carlo approach with the CLUMP software, there was no significant difference in allele distribution between the patients and controls (T1 statistic: 0.82, p = 0.99; T2: 0.82, p = 0.99; T3: 0.50, p = 0.98; T4: 0.35, p = 0.99).

Luciferase Reporter Gene Assay

Figure 1 shows observed RLE levels for two major alleles of the dinucleotide repeat polymorphism of the NTF3 gene [the A5 allele of $(CA)_{21}$ and the A3 of $(CA)_{23}$], compared to RLE without insertion of such alleles (empty pGL3-promoter vector). For all the cell lines examined (HeLa, IMR-32, and Hs683), RLE was significantly increased ($p < 0.001$) due to insertion of the polymorphic region for both alleles [$(CA)_{21}$ and $(CA)_{23}$] compared to

Fig. 1. RLE levels are shown for the major two alleles of dinucleotide repeat polymorphism of the NTF3 gene [the A5 allele of $(CA)_{21}$ and the A3 of $(CA)_{23}$], compared to RLE level without insertion of these alleles (empty pGL3-promoter vector). Error bars represent SD. *** $p < 0.001$. **a** HeLa cells. **b** IMR-32 cells. **c** Hs683 cells.

Table 1. Allele distributions of the dinucleotide repeat polymorphism of the NTF3 gene among patients with bipolar disorder and controls

	n	A0 CA_{26}	A1 CA_{25}	A2 CA_{24}	A3 CA_{23}	A4 CA_{22}	A5 CA_{21}	A6 CA_{20}	A7 CA_{19}
Patients	176	1 0.6%	6 3.4%	5 2.8%	60 34.1%	11 6.3%	71 40.3%	14 8.0%	8 4.5%
Controls	196	1 0.5%	6 3.1%	5 2.6%	69 35.2%	16 8.2%	77 39.3%	13 6.6%	9 4.6%

the empty pGL3-promoter vector; whilst there was no significant difference in RLE between the $(CA)_{21}$ and $(CA)_{23}$ alleles.

Discussion

Against our study hypothesis, we obtained no evidence for an association between bipolar disorder and the CA repeat polymorphism of the NTF3 gene in our sample. Since the sample size (88 patients and 98 controls) was not very large, we may have obtained a false negative result due to the lack of statistical power. However, the observed allele frequencies were quite similar for the patients and controls; therefore, the possibility of type II error might be minimal. Considering the previous reports of positive association between the NTF3 gene and schizophrenia [5–9], the polymorphism might have differential effects on susceptibility to schizophrenia and bipolar disorder.

The second hypothesis that we tested in the present study was that the polymorphic region of the NTF3 gene may have functional effects on transcriptional activity in an allele-dependent manner. Since the 'A3' allele was significantly increased, whilst the most frequent allele 'A5' was decreased, in schizophrenics as compared to controls [5–9], we examined these two major alleles for possible effects on the transcriptional activity by using luciferase reporter gene assay. Experiments for three cell lines yielded quite consistent results. Insertion of the polymorphic region resulted in increased RLE, compared to that without such an insertion to the pGL3-pomoter vector, suggesting that the polymorphic region may have an enhancer-like effect on the transcriptional activity. However, there was no significant difference in RLE for either cell line between the A3 and A5 alleles, which does not support our study hypothesis. Although our results cannot entirely exclude the possibility that the alleles may have differential effects on transcriptional activity in other cell lines and conditions of cell culture, our results from three cell lines suggest that there is little difference in effect on transcriptional activity between alleles. Thus the previously reported association between this polymorphism and schizophrenia may have arisen by linkage disequilibrium of the dinucleotide repeat polymorphism with other functional polymorphism.

In conclusion, our results suggest that the examined polymorphic region might have an enhancer-like effect on the transcriptional activity of the NTF-3 gene. However, we obtained no evidence for allele-dependent differential effects of the polymorphic region of the NTF-3 gene on its transcriptional activity or susceptibility to bipolar disorder.

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No Association Found between the Type 1 Sigma Receptor Gene Polymorphisms and Methamphetamine Abuse in the Japanese Population

A Collaborative Study by the Japanese Genetics Initiative for Drug Abuse

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ABSTRACT: **It has been suggested that individual genetic factors are involved in susceptibility to drug dependence and the manifestation of drug-induced psychosis. The aim of this study was to examine the relation between methamphetamine abusers/psychosis and the type 1 sigma receptor gene polymorphisms. Subjects comprised 143 MAP abusers and 181 healthy controls. Two polymor-**

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phisms in the type 1 sigma receptor gene, GC-241-240TT and A61C (Gln2Pro), were examined in the present study. No significant differences were observed in either polymorphism between healthy controls and MAP abusers/psychosis. In the subgroup analyses, the rate of CC genotype of A61C tended to be higher in MAP patients who had experienced spontaneous relapse without MAP use than in those who had not (P = .06, OR = 3.02 95%CI = 0.92–9.92). However, the level of this significant trend did not remain after the Bonferroni's multiple correction. This study suggests that type 1 sigma receptor gene is unlikely to play a major role in substance abuse liability and/or the development of MAP psychosis.

KEYWORDS: **type 1 sigma receptor gene; methamphetamine psychosis; drug dependence; Japanese; polymorphism**

INTRODUCTION

From the results of family, twin, and adoption studies, genetic factors have been reported to be involved in the susceptibility to drug dependence and the manifestation of drug-induced psychosis.[1–4] Of the compounds that induce drug dependence, methamphetamine (MAP) is one of the most widely used psychostimulants in Japan. MAP induces a strong psychological dependence, and its repeated consumption is known to result frequently in the development of psychiatric symptoms that resemble the paranoid type of schizophrenia.[5]

Sigma receptors are unique binding sites that interact with a variety of psychotomimetic drugs, including cocaine and amphetamine. Several subtypes are present in high densities in the limbic structures as well as in motor-related areas of the central nervous system. Although the exact physiological functions of the sigma receptors remain to be clarified, several lines of evidence suggest the possible involvement of sigma receptors in the development of psychosis.[6] The initial suggestion of this emerged from the observations that several of the earliest sigma ligands induced psychiatric symptoms such as delusions, hallucinations, and depersonalization. This link was later reinforced with the demonstration that several antipsychotic drugs such as haloperidol have a high affinity for sigma receptors. In addition, it has been reported that the sigma binding sites in the brain are significantly decreased in schizophrenic patients,[7] whose psychotic symptoms are similar to those seen in MAP psychosis. Therefore, it can be hypothesized that the sigma receptor gene is an important candidate gene for schizophrenia and psychostimulant-induced psychosis. The type 1 sigma receptor gene is located on human chromosome 9p13 and contains four exons.[8] Two polymorphisms—GC-241-240TT in the 5′ flanking region and A61C (Gln2Pro) in exon 1—have been identified in this gene.[9,10]

In this study, the relation between the type 1 sigma receptor gene polymorphisms and MAP abusers/psychosis was examined in the Japanese population to investigate a possible genetic influence of the type 1 sigma receptor gene polymorphisms on the development of MAP abusers/psychosis. This study was carried out as one of the collaborative studies by the Japanese Genetics Initiative for Drug Abuse (JGIDA) organized to facilitate the sample collection of MAP abusers/psychosis to investigate genetic factors related to the substance abuse liability and the characteristics of MAP psychosis.

SUBJECTS AND METHODS

Ethical Considerations

This study was initiated after the approval of the ethical committee in each attending institution of JGIDA. Written informed consent was obtained from all participating patients.

Subjects

The subjects consisted of 143 unrelated patients with MAP dependence and psychotic disorder (112 males and 31 females, average age 36 years, range 19–69 years) who met the ICD-10-DCR criteria (F15.2 and F15.5) and who were outpatients or inpatients of psychiatric hospitals of JGIDA; 183 age-, gender-, and geographical origin–matched unrelated healthy controls (143 males and 40 females, age 37 years, range 19–73 years) mostly consisted of medical staff who had no past history and no family history of drug dependence or psychotic disorders. All subjects were Japanese, born and living in areas restricted to Japan including northern Kyusyu, Setouchi, Toukai, and Kantou.

Clinical Characteristics

The patients with MAP psychosis were divided into two subgroups according to the following clinical characteristics: age at first MAP use, duration of MAP use until onset of psychosis, prognosis of psychosis (transient type vs. prolonged type), and presence or absence of the spontaneous relapse without MAP use. The rationales of why we focus on these clinical features were described previously.[11] In dividing the subgroups, the median values were adopted as the cutoff point.

Genomic Procedure

Genomic DNA was extracted from the leukocyte nuclei of peripheral blood. The type 1 sigma receptor gene polymorphisms examined in this study were GC-241-240TT in the 5′ flanking region and A61C (Gln2Pro) in exon 1. Genotyping was carried out according to the standard protocol, slightly modified from the published methods.[9,10] Briefly, each target segment was amplified by the polymerase chain reaction (PCR) method, and the PCR products were digested with the restriction enzymes, and then electrophoresed.

Statistics

The presence of the Hardy-Weinberg equilibrium was tested using a chi-square goodness-of-fit test. Comparisons of allele and genotype frequencies were carried out using the chi-squared test for 2×2 and 2×3 contingency tables. Significance level and significant trend level were defined when the P value was less than .05 and .1, respectively.

TABLE 1. Genotype and allele frequencies of the type 1 sigma receptor gene polymorphisms in MAP abusers/psychosis and healthy controls

(a) GC-241-240TT in the 5′ flanking region

	Genotype[a]				Allele[a]		
Group	GC/GC	GC/TT	TT/TT	*P* Value[b]	GC	TT	*P* Value[b]
Controls	90	78	13		258	104	
(*n* = 181)	49.7%	43.1%	7.2%		71.3%	28.7%	
MAP abusers	70	61	12	0.92	201	85	0.78
(*n* = 143)	49.0%	42.7%	8.4%		70.3%	29.7%	
MAP psychosis	56	50	12	0.66	162	74	0.49
(*n* = 118)	47.5%	42.4%	10.2%		68.6%	31.4%	

(b) A61C (Gln2Pro) in exon 1

	Genotype[a]				Allele[a]		
Group	AA	AC	CC	*P* Value[b]	A	C	*P* Value[b]
Controls	86	83	14		255	111	
(*n* = 181)	47.0%	45.4%	7.7%		69.7%	30.3%	
MAP abusers	61	65	17	0.40	187	99	0.25
(*n* = 143)	42.7%	45.5%	11.9%		65.4%	34.6%	
MAP psychosis	49	55	14	0.39	153	83	0.77
(*n* = 118)	41.5%	46.6%	11.9%		64.8%	35.2%	

[a]*Upper row*, number of subjects; *lower row*, frequency.
[b]Compared with controls.

RESULTS

The genotype distribution was not significantly different from that expected according to the Hardy-Weinberg equilibrium in any group and in any subgroup examined in the present study.

The genotypic distribution and the allelic frequency of two polymorphisms—that is, GC-241-240TT in the 5′ flanking region and A61C (Gln2Pro) in exon 1, in the type 1 sigma receptor gene between healthy controls and the patients with MAP abusers/psychosis—are shown in TABLE 1. No significant differences were observed in either polymorphism between healthy controls and MAP abusers/psychosis.

The genotypic distribution and the allelic frequency of two polymorphisms, that is, GC-241-240TT in the 5′ flanking region and A61C (Gln2Pro) in exon 1, in the type 1 sigma receptor gene between the subgroups in patients with MAP psychosis, are shown in TABLE 2 and TABLE 3, respectively.

In the subgroup analyses, no significant differences were observed in genotype, allele, or homozygosity frequencies between any subgroup of the patients with MAP psychosis. Although the rate of CC genotype of A61C tended to be higher in MAP patients who have experienced spontaneous relapse without MAP use than in those who have not ($P = .06$, OR = 3.02 95%CI = 0.92–9.92), this significant trend level did not remain after the Bonferroni's multiple correction.

TABLE 2. Genotype and allele frequencies of the GC-241-240TT polymorphism in the type 1 sigma receptor gene between subgroups in MAP psychosis

Subgroups in MAP psychosis	Genotype[a]				Allele[a]		
	GC/GC	GC/TT	TT/TT	*P* Value[b]	GC	TT	*P* Value
(a) Age at first MAP use (in years)							
≧ 20	27	25	4		79	33	
(*n* = 56)	48.2%	44.6%	7.1%	0.64	70.5%	29.5%	0.63
< 20	27	23	7		77	37	
(*n* = 57)	47.4%	40.4%	12.3%		67.5%	32.5%	
(b) Duration of MAP use until onset of psychosis							
≧ 3 years	29	22	4		80	30	
(*n* = 55)	52.7%	40.0%	7.3%	0.30	72.7%	27.3%	0.13
< 3 years	18	23	6		59	35	
(*n* = 47)	38.3%	48.9%	12.8%		62.8%	37.2%	
(c) Disappearance type psychosis[b]							
Transient type	31	28	6		90	40	
(*n* = 65)	47.7%	43.1%	9.2%	0.94	69.2%	30.8%	0.73
Prolonged type	21	21	5		63	31	
(*n* = 47)	44.7%	44.7%	10.6%		67.0%	33.0%	
(d) Spontaneous relapse without MAP use							
Present	21	16	6		58	28	
(*n* = 43)	48.8%	37.2%	14.0%	0.39	67.4%	32.6%	0.72
Absent	33	33	5		99	43	
(*n* = 71)	46.5%	46.5%	7.0%		69.7%	30.3%	

[a] *Upper row*, number of subjects; *lower row*, frequency.
[b] *Transient type*: psychotic symptoms improved within one month after discontinuation of METH, along with initiation of treatment with neuroleptics; *prolonged type*: psychotic symptoms continued for more than one month even after discontinuation of METH, along with initiation of treatment with neuroleptics.

DISCUSSION

The present results did not show any allelic association between the type 1 sigma receptor gene polymorphisms and the patients with MAP abusers/psychosis. Moreover, no allelic association with any of the subgroup analyses on the clinical characteristics examined in the present study was detected, indicating that the type 1 sigma receptor gene is unlikely to play a major role in substance abuse liability and/or development of MAP psychosis.

As far as we know, this is the first report on the association study between the type 1 sigma receptor gene polymorphism and the patients with MAP psychosis, whose psychiatric symptoms are similar to those observed in patients with schizophrenia. There are two studies examining the association between the type 1 sigma receptor

TABLE 3. Genotype and allele frequencies of the A61C (Gln2Pro) polymorphism in the type 1 sigma receptor gene between subgroups in MAP psychosis

Subgroups in MAP psychosis	Genotype[a] AA	AC	CC	*P* Value	Allele[a] A	C	*P* Value
(a) Age at first MAP use (in years)							
≧ 20	24	27	5		75	37	
(*n* = 56)	42.9%	48.2%	8.9%	0.68	67.0%	33.0%	0.64
< 20	24	25	8		73	41	
(*n* = 57)	42.1%	43.9%	14.0%		64.0%	36.0%	
(b) Duration of MAP use until onset of psychosis							
≧ 3 years	26	24	5		76	34	
(*n* = 55)	47.3%	43.6%	9.1%		69.1%	30.9%	
< 3 years	16	24	7	0.35	56	38	0.16
(*n* = 47)	34.0%	51.1%	14.9%		59.6%	40.4%	
(c) Disappearance type psychosis[b]							
Transient type	27	30	8		84	46	
(*n* = 65)	41.5%	46.2%	12.3%		64.6%	35.4%	
Prolonged type	19	23	5	0.94	61	33	0.97
(*n* = 47)	40.4%	48.9%	10.6%		64.9%	35.1%	
(d) Presence or absence of the spontaneous relapse without MAP use							
Yes	19	16	8		54	32	
(*n* = 43)	44.2%	37.2%	18.6%		62.8%	37.2%	
No	30	36	5	0.12	96	46	0.46
(*n* = 71)	42.3%	50.7%	7.0%		67.6%	32.4%	

[a] *Upper row*, number of subjects; *lower row*, frequency.
[b] *Transient type*: psychotic symptoms improved within one month after discontinuation of METH along with initiation of treatment with neuroleptics; *prolonged type*: psychotic symptoms continued for more than one month even after discontinuation of METH, along with initiation of treatment with neuroleptics.

gene polymorphisms and schizophrenia in the Japanese population. Ishiguro *et al.*[9] first observed a significant association between the presence of the TT/Pro2 haplotype and schizophrenia (odds ratio = 1.27, $P = .04$), and they suggested a possible role in the pathogenesis of schizophrenia. Subsequently, Ohmori *et al.*[10] also observed a weak association between homozygosity for TT/Pro2 and schizophrenia ($P = .045$). However, they reported that this significance did not remain when a Bonferroni's correction was made ($P = .135$) and concluded that the type 1 sigma receptor gene is unlikely to play a major role in the pathogenesis of schizophrenia.

However, considering the previous reports on the close relationship between sigma receptor and psychiatric symptoms, the possibility of some involvement in the sigma receptor gene polymorphism for the development of psychiatric conditions such as MAP psychosis and schizophrenia cannot be completely ruled out. Moreover, because the sample size for the present study is fairly small, there is the possi-

bility of a type II error to detect the significant difference. Therefore, further research is needed to clarify the exact role of these polymorphic sites in determining certain phenotypes, using a larger number of samples, that can be related to some forms of psychosis.

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ORIGINAL ARTICLES

Association Between Chromogranin B Gene Polymorphisms and Schizophrenia in the Japanese Population

Yoshimi Iijima, Toshiya Inada, Tsuyuka Ohtsuki, Hisashi Senoo, Masaki Nakatani, and Tadao Arinami

Background: *We found in previous work a significant association between schizophrenia and D20S95 on chromosome 20p12.3. In this study, we analyzed 10 microsatellite markers and found an association of schizophrenia with D20S882 and D20S905 that flank D20S95. The chromogranin B gene (*CHGB*) is 30 kb from D20S905. The chromogranin B (secretogranin 1) belongs to a series of acidic secretory proteins that are widely expressed in endocrine and neuronal cells, and its cerebrospinal fluid levels have been reported to decrease in patients with chronic schizophrenia.*

Methods: *We screened for polymorphisms in* CHGB *with polymerase chain reaction direct sequencing methods in 24 Japanese schizophrenic patients and identified a total of 22 polymorphisms. Allelic and genotypic distributions of detected polymorphisms were compared between unrelated Japanese schizophrenic patients (*n = *192) and healthy control subjects (*n = *192).*

Results: *Statistically significant differences in the allelic distributions were found between schizophrenic patients and control subjects for 1058C/G (A353G) (corrected* $p = 7.7 \times 10^{-5}$*) and 1104A/G (E368E) (corrected* $p = 8.1 \times 10^{-6}$*). The 1058C/G and 1104A/G alleles were in almost complete linkage disequilibrium and were in linkage disequilibrium with D20S95.*

Conclusions: *Results suggest that the* CHGB *variations are involved in the susceptibility to schizophrenia in our study population.*

Key Words: Schizophrenia, chromogranin B, association study, neuropeptide

The region of 20p12.3-p11 may contain a locus of predisposition to schizophrenia (Lewis et al 2003). In our previous screening association study, we observed a significant association with schizophrenia at the locus of D20S95 ($p = 5 \times 10^{-6}$, corrected p value after Bonferroni correction, .00035) on 20p12.3 (Kitao et al 2000). The marker D20S95 is approximately 2 megabases (Mb) outside the 21.2 to 47.5 cM region of chromosome 20 highlighted in the meta-analysis of Lewis et al (2003), though quite possibly within its confidence bounds. In the individual genome scans, Moises et al (1995) reported a p-value of .009 with marker D20S40 and Hovatta et al (1998) found a maximum lod score of 1.22 with marker D20S172. These markers are located approximately 7 Mb and 12 Mb centromeric to D20S95.

The only known gene within 180 kb from D20S95 is the gene encoding chromogranin B, a tyrosine-sulfated secretory protein found in a wide variety of peptidergic endocrine cells. The granins (secretogranins/chromogranins) belong to a family of soluble proteins stored and released from the large dense-core secretory vesicles of the synapse (Benedum et al 1987; Winkler and Fischer-Colbrie 1992). A number of studies have compared the chromogranin levels in cerebrospinal fluid (CSF) between healthy control subjects and schizophrenic patients at various stages of this disease (Landen et al 1999; Miller et al 1996; van Kammen et al 1991, 1992, 1994). Landen et al (1999) reported that levels of chromogranin A and chromogranin B were lower in chronic schizophrenic patients and pointed out the possibility that an acute increase of chromogranin levels reflects an active disease process and a chronic decline indicates an advanced neurodegenerative process. Reduction of chromogranin B-like immunoreactivity in distinct subregions of the hippocampus from individuals with schizophrenia was reported (Nowakowski et al 2002). An association between some *CHGB* polymorphisms and schizophrenia was reported in the Chinese Han population (Zhang et al 2002).

In the present study, we performed additional dense-mapping analyses using 10 microsatellite markers close to D20S95 and the mutation search of the *CHGB*, followed by the case-control association studies on its detected polymorphisms in schizophrenic patients, using the same sample set as we previously used in the screening study in which a significant association with schizophrenia was detected at D20S95.

Methods and Materials

Ethical Considerations

The present study was approved by the Ethical Committee of the Kohnodai area, National Center of Neurology and Psychiatry, University of Tsukuba, and Nagoya University. Written informed consent was obtained from all subjects.

Subjects

The DNA samples were all selected from those of the subjects enrolled in our previous screening study (Kitao et al 2000). The subjects consisted of 192 schizophrenic patients and 192 healthy control subjects. The schizophrenic patients (91 men, 101 women; aged 19–90 years [mean 57.2 years]) were all inpatients recruited from several psychiatric facilities located around the Tokyo area. They were interviewed several times during a hospitalization period of 6 months or more by experienced psychiatrists who were familiar with the structured clinical interview for DSM-III-R (SCID) rating system (Spitzer et al 1992). The diagnosis of schizophrenia was assigned on the basis of clinical interviews and chart review of medical records, accord-

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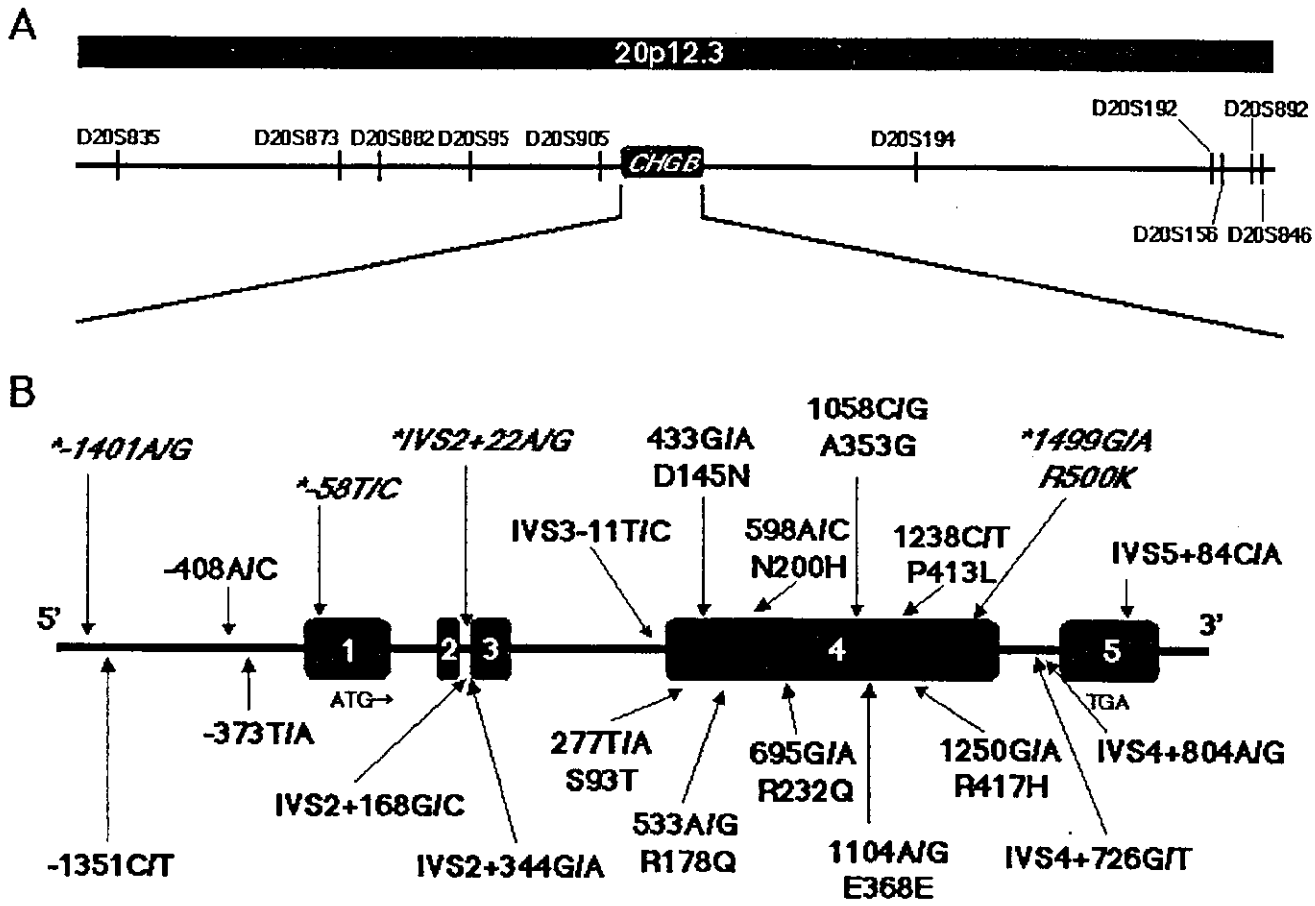

Figure 1. **(A)** The positions of the microsatellite markers in relation to chromogranin B gene (*CHGB*). **(B)** Genomic organization and positions of the SNPs in *CHGB*. *Italics are newly identified SNPs. SNP, single nucleotide polymorphism.

Table 1. Results of the Additional Dense-Mapping Analyses Using 10 Microsatellite Markers Close to D20S95

Microsatellite Markers[a]	Distance from *CHGB* (kb)	Number of Subjects		Statistics[b]	
		Schizophrenia	Control	H-W[c]	S vs. C[d]
D20S835	−577	172	192	ns	ns
D20S873	−295	175	191	ns	ns
D20S882	−257	203	196	.02	.02
D20S95	−176	230	217	ns	.002
D20S905	−29	184	190	ns	.04
CHGB	0				
D20S194	251	164	135	ns	ns
D20S192	805	171	192	ns	ns
D20S156	809	171	192	ns	ns
D20S892	858	174	192	ns	ns
D20S846	873	176	192	ns	ns

Details are shown in Appendix 1.

CHGB, chromogranin B gene; kb, kilobase; H-W, Hardy-Weinberg equilibrium; S, schizophrenia; C, control.

[a]These markers are listed in the order from telomeric (D20S835) to centromeric (D20S846) site. The average distance between the markers is about 120 kb.

[b]*p* value is denoted when $p < .05$.

[c]Deviations from the Hardy-Weinberg equilibrium.

[d]Comparison between schizophrenia vs. control (empirical *p*).

ing to the DSM-III-R criteria (American Psychiatric Association 1987). We actually did not use SCID itself at the clinical interview because: (1) a validated Japanese translation version of SCID was not available at the time of clinical evaluation in this study, and (2) we considered that diagnosis by repeated interviews by trained attendant physicians seems to be more reliable than SCID performed by a nonattendant physician only at once. Control subjects (96 men, 96 women; aged 24–87 years [mean 49.8 years]) were recruited mostly from the medical staff working in the psychiatric facilities and had no history of psychoses. All subjects were of Japanese descent, born to Japanese parents. The sampling methods of the subjects were satisfied with the criteria of the proposed checklists for gene-disease association study (Colhoun et al 2003; Little et al 2002).

Genomic Procedure

Genomic DNA was extracted from leukocytes in venous blood samples. Ten microsatellite markers on chromosome 20p12.3, D20S835 (Genome Database [GDB]:603368), D20S873 (GDB:609813), D20S882 (GDB:610170), D20S905 (GDB:612744), D20S916 (GDB:614700), D20S194 (GDB:200327), D20S192 (GDB:200199), D20S156 (GDB:198517), D20S892 (GDB:611556),

Table 2. Pair-Wise Linkage Disequilibrium in the *CHGB*

	4 −1401A/G	5 −1351C/T	6 −408A/C	7 −373T/A	8 −58T/C	9 IVS3-11T/C	10 277T/A	11 433G/A	12 533A/G	13 598A/C	14 695G/A	15 1058C/G	16 1104A/G	17 1238C/T	18 1250G/A	19 1499G/A
4 −1401A/G		1.00	1.00	.73	.51	.60	1.00	1.00	1.00	1.00	.50	.48	.37	.69	.84	.60
5 −1351C/T	.10		.90	.72	.43	1.00	.89	1.00	.98	.85	.92	.64	.64	.57	.58	.93
6 −408A/C	.01	.35		1.00	.96	.77	.86	.45	.88	1.00	.67	.84	.84	.77	.83	.90
7 −373T/A	.00	.09	.27		.58	1.00	.72	.85	.76	.53	.51	.68	.70	.55	1.00	1.00
8 −58T/C	.00	.02	.45	.01		.50	.36	.44	.42	.74	.43	.64	.63	1.00	.66	1.00
9 IVS3-11T/C	.00	.07	.07	.02	.00		1.00	1.00	1.00	1.00	.98	.61	.62	.83	.74	.51
10 277T/A	.01	.70	.23	.07	.01	.06		.99	.99	1.00	.90	.58	.57	.56	.51	.92
11 433G/A	.01	.38	.20	.08	.08	.17	.37		1.00	1.00	.92	.47	.46	.23	.43	.67
12 533A/G	.02	.58	.49	.14	.04	.11	.60	.60		1.00	.94	.63	.62	.32	.42	.83
13 598A/C	.13	.06	.10	.02	.04	.01	.08	.07	.12		1.00	.79	.79	.54	.43	.90
14 695G/A	.00	.06	.06	.01	.02	.87	.06	.16	.09	.01		.58	.59	.74	.69	.48
15 1058C/G	.00	.13	.57	.16	.29	.05	.08	.15	.21	.04	.02		1.00	1.00	.98	.77
16 1104A/G	.00	.13	.57	.16	.29	.05	.08	.15	.21	.04	.04	1.00		1.00	.98	.77
17 1238C/T	.02	.04	.08	.02	.04	.00	.04	.00	.01	.29	.00	.10	.10		.75	.81
18 1250G/A	.01	.25	.34	.11	.19	.04	.16	.12	.17	.02	.03	.66	.66	.03		.82
19 1499G/A	.01	.05	.05	.02	.03	.31	.04	.09	.07	.01	.24	.10	.10	.01	.04	

Upper diagonal figures are *D'* and lower diagonal figures are r^2.
CHGB, chromogranin B gene.

and D20S846 (GDB:606163), were amplified by polymerase chain reaction (PCR). The positions of these markers in relation to *CHGB* is shown in Figure 1A. The PCR primers are available on the GDB (http://gdbwww.gdb.org). Genotyping to determine the lengths of the microsatellite alleles was done with an ABI PRISM 3100 DNA sequencer (Applied Biosystems, Foster City, California). The sequence and genomic structure of *CHGB* were obtained from the high throughput genome sequences (HTGs) database of the Blast server at the National Center for Biotechnology Information (NCBI) (http://www.ncbi.nlm.nih.gov/BLAST/). All exons containing exon-intron junctions and a 5'-side regulatory region of *CHGB* were amplified by the PCR method. The primers are available on request. Polymorphisms were screened in 24 schizophrenic patients by direct sequencing of PCR products with the Big Dye Terminator Cycle Sequencing kit (Applied Biosystems, Foster City, California) and on the ABI PRISM 3100 DNA sequencer. All nucleotide variants detected in this study were genotyped by direct sequencing after PCR amplification. Trace data were aligned by Sequencher software (Gene Codes Corp, Ann Arbor, Michigan) and scanned for polymorphisms.

Statistical Procedures

Microsatellite Marker Analyses. Assessing the deviations from Hardy-Weinberg equilibrium and the case-control comparison of the distribution of the microsatellite allele frequencies between control subjects and schizophrenic patients were performed by the program implemented in MEGA2 (Guo and Thompson 1992).

Single Nucleotide Polymorphism Analyses. Deviations of the genotype distributions from the Hardy-Weinberg equilibrium were assessed with the 2×3 χ^2 test. Case-control comparisons of genotype and allele frequencies of polymorphisms were done with the Armitage Trend Test and 2×2 χ^2 tests. We examined associations by permutation procedure in COCAPHASE ver 2.4 to determine the empirical significance of our findings. We did not use -EM and -missing options. The χ^2 value, the p-value, and the odds ratio (OR) were calculated with the SPSS computer package for Windows Release 11.0J (SPSS Japan Inc., Tokyo, Japan). Linkage disequilibrium analyses were conducted with the Cocaphase program: unbiased application of the transmission/disequilibrium test to multilocus haplotypes (Dudbridge et al 2000).

Results

Results of the additional dense mapping association analyses using 10 microsatellite markers close to D20S95 on 20p12.3 with schizophrenia are shown in Table 1 and supplemental data table 1. The genotype frequencies of these microsatellite markers did not significantly deviate from Hardy-Weinberg equilibrium except for D20S882 ($P = 0.02$). As shown in Table 1, possible association with schizophrenia was detected in the loci of D20S882 and D20S905, in addition to D20S95. The 109-bp allele of D20S95 was more frequent in the patient group (.10) than in the control group (.03, uncorrected $p = 7.2 \times 10^{-5}$, empirical $p = .002$). The 80-bp allele of D20S882 was less frequent in the patient group (.14) than in the control group (.22, uncorrected $p = .0009$, empirical $p = .02$). The 90-bp allele of D20S905 was less frequent in the patient group (.01) than in the control group (.05, uncorrected $p = .002$, empirical $p = .004$). Although there was a global significance for the haplotype comprising the three microsatellites ($p = 1 \times 10^{-9}$), no specific individual haplotype of these microsatellite markers was significantly associated with schizophrenia after correction for the number of haplotypes.

Table 3. Genotype and Allelic Distribution of the *CHGB* SNPs in Japanese Control Subjects and Patients with Schizophrenia

		Genotype					Allele			
					p Value[a]					
Polymorphisms Group	*N*	Genotype Count			H-W[c]	S vs. C[d]	Count (frequency)		Odds Ratio (95% CI)	*p* Value[b] S vs. C[d]
−1401A/G		AA	AG	GG			A	G		
Control	192	186	6	0	1.00	.79	378 (.98)	6 (.02)	1.22 (.40–3.65)	.73
Schizophrenia	185	178	7	0	1.00		363 (.98)	7 (.02)		
−1351C/T		CC	CT	TT			C	T		
Control	192	78	77	37	.30	.87	233 (.61)	151 (.39)	.92 (.69–1.23)	.58
Schizophrenia	186	72	89	25	.98		233 (.63)	139 (.37)		
−408A/C		AA	AC	CC			A	C		
Control	96	14	45	37	1.00	.47	73 (.38)	119 (.62)	.79 (.53–1.19)	.26
Schizophrenia	95	15	53	27	.67		83 (.44)	107 (.56)		
−373T/A		TT	TA	AA			T	A		
Control	94	71	21	2	.99	.43	163 (.87)	25 (.13)	.99 (.55–1.79)	.97
Schizophrenia	95	70	25	0	.33		165 (.87)	25 (.13)		
−58T/C		TT	TC	CC			T	C		
Control	185	106	67	12	.96	.24	279 (.75)	91 (.25)	1.22 (.88–1.69)	.24
Schizophrenia	181	94	71	16	.95		259 (.72)	103 (.28)		
IVS3-11T/C		TT	TC	CC			T	C		
Control	187	154	32	1	.84	.32	340 (.91)	34 (.09)	.66 (.38–1.14)	.14
Schizophrenia	186	164	21	1	.99		349 (.94)	23 (.06)		
277T/A (S93T)		TT	TA	AA			T	A		
Control	189	73	86	30	.91	.82	232 (.61)	146 (.39)	.94 (.70–1.26)	.68
Schizophrenia	187	73	89	25	.98		235 (.63)	139 (.37)		
433G/A (D145N)		GG	GA	AA			G	A		
Control	187	73	90	24	.96	.78	236 (.63)	138 (.37)	.99 (.73–1.33)	.94
Schizophrenia	187	77	83	27	.91		237 (.63)	137 (.37)		
533A/G (R178Q)		AA	AG	GG			A	G		
Control	188	51	90	47	.92	.35	192 (.51)	184 (.49)	.87 (.65–1.16)	.34
Schizophrenia	187	42	94	51	1.00		178 (.48)	196 (.52)		
598A/C (N200H)		AA	AC	CC			A	C		
Control	189	149	39	1	.82	.59	337 (.89)	41 (.11)	1.12 (.72–1.76)	.61
Schizophrenia	187	143	43	1	.57		329 (.88)	45 (.12)		
695G/A (R232Q)		GG	GA	AA			G	A		
Control	186	157	28	1	1.00	.45	342 (.92)	30 (.08)	.87 (.50–1.50)	.61
Schizophrenia	184	161	20	3	.50		342 (.93)	26 (.07)		
1058C/G (A353G)		CC	CG	GG			C	G		
Control	192	49	89	54	.80	1.3×10^{-5}	187 (.49)	197 (.51)	1.96 (1.46–2.63)	5.9×10^{-6}
Schizophrenia	190	21	82	87	.97		124 (.33)	256 (.67)		
1104A/G (E368E)		AA	AG	GG			A	G		
Control	192	49	89	54	.80	2.0×10^{-6}	187 (.49)	197 (.51)	2.11 (1.57–2.84)	6.2×10^{-7}
Schizophrenia	187	20	76	91	.89		116 (.31)	258 (.69)		
1238C/T (P413L)		CC	CT	TT			C	T		
Control	192	155	36	1	.82	.54	346 (.90)	38 (.10)	1.15 (.72–1.83)	.55
Schizophrenia	187	147	38	2	1.00		332 (.89)	42 (.11)		
1250G/A (R417H)		GG	GA	AA			G	A		
Control	192	71	84	37	.67	.006	226 (.59)	158 (.41)	1.51 (1.13–2.01)	.005
Schizophrenia	187	42	98	47	.90		182 (.49)	192 (.51)		
1499G/A (R500K)		GG	GA	AA			G	A		
Control	191	160	29	2	.84	.270	349 (.91)	33 (.09)	.67 (.38–1.16)	.15
Schizophrenia	186	166	18	2	.75		350 (.94)	22 (.06)		

CHGB, chromogranin B gene; H-W, Hardy-Weinberg equilibrium; S, schizophrenia; C, control; CI, confidence interval.
[a]Armitage's trend test.
[b]Chi-squared test.
[c]H-W: Observed genotype vs. expected genotype according to Hardy-Weinberg equilibrium.
[d]S vs. C: Genotype comparison between schizophrenia vs. control.

All exons containing exon-intron junctions and a 5′-side regulatory region of *CHGB* were screened for polymorphisms in 24 schizophrenic patients. A total of 22 single nucleotide polymorphisms (SNPs) were identified (Figure 1B). Eighteen SNPs, −1351C/T (rs236139), −408A/C (rs236140), −373T/A (rs236141), IVS2+168G/C (rs236145), IVS2+344G/A (rs236146), IVS3−11T/C (rs6139872), 277T/A(S93T) (rs6085324), 433G/A(D145N) (rs6133278), 533G/A(R178Q) (rs910122), 598A/C(N200H) (rs881118), 695G/A(R232Q) (rs6139873), 1058G/C(A353G) (rs236152), 1104G/A(E368E) (rs236153), 1238C/

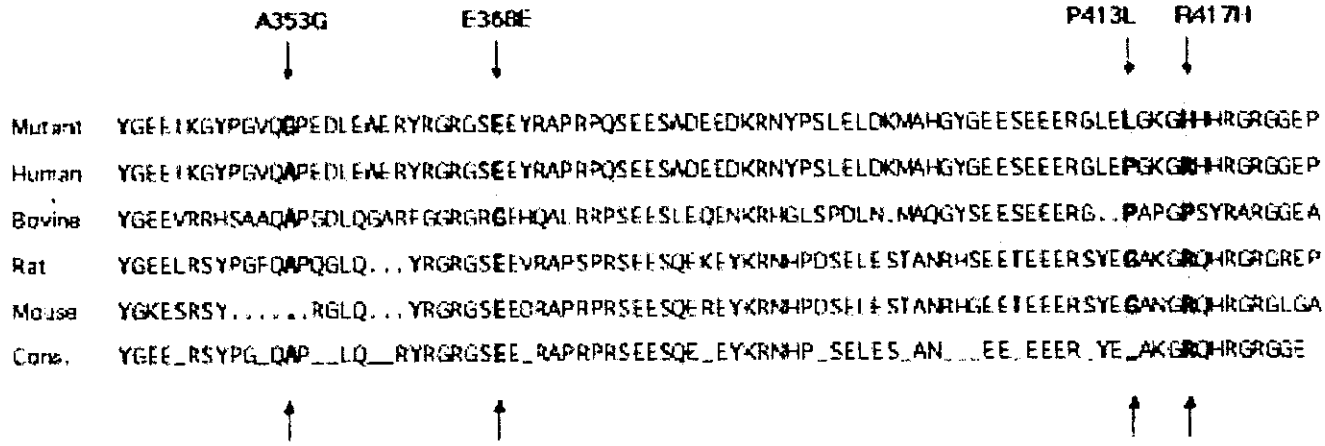

Figure 2. Comparison of the amino acid sequences of chromogranin B from human, bovine, rat, and mouse. Cons. is calculated consensus sequence. Dots (.) denote deletions in the sequence.

T(P413L) (rs742710), 1250G/A(R417H) (rs742711), IVS4+726G/T (rs236154), IVS4+804G/A (rs236155), and IVS5+84C/A (rs2821), are listed in the database for single nucleotide polymorphisms (dbSNP) in the NCBI (http://www.ncbi.nlm.nih.gov/SNP/). Three SNPs, −1401A/G, IVS2+22A/G, and 1499G/A(R500K), not listed in the dbSNP database were identified in the mutation search of *CHGB* in the Chinese Han population (Zhang et al 2002). Seven SNPs, −1095G/C (rs989462), −1042G/A (rs6085321), 402G/C(K177N) (rs236150), 727G/A(T423A) (rs236151), 1466C/T(L489L) (rs6107717), IVS4+9T/C (rs617000), and IVS5+277A/T (rs 236156), which are listed in the dbSNP database, were not detected in the Japanese population. One SNP, −58T/C, was newly identified in this study. Among these SNPs, 16 at the coding or regulatory regions were selected for further association study.

Linkage disequilibrium was found in many pairs of the 16 SNPs (Table 2). The genotype and allele frequencies of all SNPs are shown in Table 3. The genotype distributions did not deviate significantly from those expected according to the Hardy-Weinberg equilibrium for polymorphisms. Statistically significant differences in allele distributions were found between schizophrenic patients and healthy control subjects for 1058C/G ($p = 5.9 \times 10^{-6}$, empirical $p = 8.2 \times 10^{-5}$, OR = .510, 95% confidence interval [CI] = .38–.68) and 1104A/G ($p = 6.2 \times 10^{-7}$, empirical $p = 1 \times 10^{-5}$, OR = .474, 95% CI = .35–.64), and a trend of association was found between 1250G/A and schizophrenia ($p = .005$, empirical $p = .05$, OR = 1.51, 95% CI = 1.13–2.01) (Table 2). The genotype distributions of these three SNPs suggest the presence of a gene dose-response effect: a linear trend in disease risk with number of copies of the high-risk allele (uncorrected $p = 1.3 \times 10^{-5}$, uncorrected $p = 2.0 \times 10^{-6}$, and uncorrected $p = .006$, respectively). The frequency of the risk GG genotype of A353G in the control subjects was .28 and its OR for schizophrenia was 2.1 (95% CI = 1.4–3.3) compared with other genotypes. Two SNPs, 1058C/G and 1104A/G, were in almost complete linkage disequilibrium (Table 2), and almost the same p-value was obtained for the haplotype associations with schizophrenia (uncorrected $p = 6.1 \times 10^{-7}$). Other haplotypes are also associated with schizophrenia; however, they are mainly due to 1058C/G and 1104A/G polymorphisms (Table 4). Linkage disequilibrium was significant ($p < .05$) between SNPs associated with schizophrenia, namely 1058C/G (D′ = .43), 1104A/G (D′ = .44), and 1250G/A (D′ = .37) in the *CHGB* and the 109-bp allele of D20S95.

Although it has been reported that the significant association with schizophrenia ($p < .001$) was observed at two SNPs of 433G/A and 533A/G in the Chinese Han population, these findings were not replicated in our present study. The power of this study to replicate the findings from the Chinese-Han poupulation was 90%.

Discussion

Our hypothesis that the chromogranin B gene (*CHGB*) is a plausible positional candidate for association with schizophrenia can be strongly supported by the results of our additional dense mapping analyses examined in the present study. This hypothesis is further supported by the findings in our subsequent association study between *CHGB* and schizophrenia: a significant association of 1058C/G and 1104A/G, and a trend of association of 1250G/A. These SNPs were in linkage disequilibrium with the 109-bp allele of D20S95. Association study may lead to false-positive and false-negative signals because of genetic stratification or population subdivision. Our subjects were all of Japanese descent living in the same area and we have no evidence of population stratification in our case or control samples. As shown in a previous report, Japanese is a relatively homogeneous population with no stratification (Daimon et al 2003; Kakiuchi et al 2003). However, unknown population stratification would not be excluded.

There is another Chinese study showing a significant association between some SNPs of *CHGB* and schizophrenia. Although our study and a study of Zang et al (2002) found significant associations between *CHGB* SNPs and schizophrenia, the associated SNPs differed between the two studies. Significant associations with schizophrenia were found at two SNPs, 433G/A ($p = .004$, the G allele was associated with schizophrenia, OR = 1.60, 95% CI = 1.17–2.18) and 533A/G ($p = .0005$, the A allele was associated with schizophrenia, OR = 1.73, 95% CI = 1.28–2.18), in the Chinese Han population (Zhang et al 2002). We failed to find these associations in the present study, 433G/A (OR = 1.01, 95% CI = .75–1.36) and 533A/G (OR = .87, 95% CI = .65–1.16). The ORs between the two studies significantly differed ($p = .04$ for 433G/A and $p = .001$ for 533G/A, respectively). The alleles

Table 4. Haplotypes Showing Most Significant Association with Schizophrenia per Each Window

Haplotype Window	433 G/A	533A/G	598A/C	695G/A	1058C/G	1104A/G	1238C/T	1250G/A	1499G/A	Haplotype Frequency (%) Schiz	Control	*p* Values Global	Permutation Global	Individual
Window 2					C	A				.31	.49	6.6×10^{-6}	.00009	6.6×10^{-6}
Window 3					C	A	C			.31	.49	5.9×10^{-5}	.0007	1.0×10^{-5}
Window 4				G	C	A	C			.27	.43	.0007	.01	2.0×10^{-5}
Window 5			A	G	C	A	C			.27	.40	.003	.04	.0001
Window 6		A	A	G	C	A	C			.03	.12	.005	.04	.002

Schiz, schizophrenia

that found to be associated with schizophrenia in the Chinese population, the G allele of the 433G/A polymorphism and the A allele of the 533A/G polymorphism, were on the haplotypes negatively associated with schizophrenia in our Japanese population. Linkage disequilibrium between 1058C/G and 433G/A or 533A/G were not observed (D′ = .47, D′ = .63, respectively). Our Japanese study tested more SNPs, and the most interesting SNPs were not tested in the larger Chinese sample. The SNPs significantly associated with schizophrenia in the Chinese population, however, also were nonsynonymous and of potential interest. Although it is possible that the apparent discrepancy between our study and that of Zhang et al (2002) occurred by chance, it is also possible that the discrepancy is due to ethnic differences or differences in the clinical characteristics of schizophrenic patients. We have observed that *CHGB* SNPs are associated with extrapyramidal side effects and clinical symptoms (unpublished data). Therefore, further studies are necessary to address the discrepancy between the Japanese and Chinese studies. Associations between *CHGB* and schizophrenia have been found in two independent studies of East Asian populations, indicating that *CHGB* is a plausible association with susceptibility to schizophrenia and is one of the genes contributing to the linkage of 20p12.3-p11 to schizophrenia found by meta-analysis (Lewis et al 2003).

The granin family plays an important role in the sorting and aggregation of secretory products in the trans-Golgi network (Ozawa and Takata 1995). Chromogranin A, chromogranin B (secretogranin I), and chromogranin C (secretogranin II) are well known. Chromogranin B also localizes to the nucleus and controls transcription of many genes, including those for transcription factors (Yoo et al 2002). Recently, chromogranin B was reported to have an important role in the intracellular calcium signaling in neurons by interacting with IP3 receptor in a pH-dependent manner (Thrower et al 2003). Human chromogranin B consists of 657 amino acids (Benedum et al 1987). Since the function of chromogranin B is still a matter of debate, it is acceptable that no functional analysis was performed for the 1058G/C(A353G). However, the amino acid 353A and 417R are relatively conserved among species, suggesting that the substitution has some impact on the protein function (Bauer and Fischer-Colbrie 1991) (Figure 2).

Because two of three SNPs we found to be associated with schizophrenia were nonsynonymous, they might be related directly to the susceptibility to schizophrenia. It will be important to replicate the finding in independent samples of schizophrenia of the Japanese population. However, it is possible that these SNPs are just in linkage disequilibrium with other polymorphisms responsible for giving the disease susceptibility. Our findings represent an initial step toward an understanding of the possible etiologic role that *CHGB* plays in schizophrenia. It will be interesting to consider the functional changes in the chromogranin B proteins derived from its genetic variations that may have roles in the clinical phenotypes of schizophrenia. Further research is needed with transgenic mice to clarify the exact role of chromogranin B in the pathophysiology of schizophrenia, especially to identify the functional changes derived from the genetic variations of *CHGB*. Clinical studies are also required to evaluate the psychopathological features in schizophrenic patients who have biochemical changes in chromogranin B levels and *CHGB* variations.

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Appendix 1. Supplemental Data for Table 1. Case-Control Association Analyses with Microsatellite Markers on Chromosome 20p

	Individual Allele					Global		
Marker	Allele (bp)	Case	Control	Chi-square	*p*	LRS	DF	*p*
D20S835						8.5	10	.58
	195	.169	.193	.712	.399			
	197	.003	.000	1.501	.221			
	199	.009	.013	.313	.576			
	201	.015	.013	.031	.861			
	203	.142	.138	.029	.864			
	205	.349	.388	1.197	.274			
	207	.087	.073	.505	.478			
	209	.017	.026	.633	.426			
	211	.119	.096	.987	.321			
	213	.087	.060	2.004	.157			
	215	.003	.000	1.501	.221			
D20S873						7.9	7	.34
	185	.000	.008	3.913	.048			
	187	.291	.270	.430	.512			
	189	.003	.010	1.685	.194			
	191	.100	.105	.044	.834			
	193	.026	.018	.466	.495			
	195	.537	.545	.040	.842			
	197	.034	.042	.288	.592			
	199	.009	.003	1.236	.266			
D20S882						21.3	7	.003
	72	.037	.031	.245	.620			
	74	.335	.247	7.420	.006			
	76	.126	.181	4.759	.029			
	78	.355	.316	1.316	.251			
	80	.136	.219	9.711	.002			
	82	.007	.005	.169	.681			
	84	.002	.000	1.353	.245			
	86	.002	.000	1.353	.245			
D20S95						116.6	11	.000
	95	.000	.002	1.148	.284			
	97	.009	.103	48.970	.000			
	99	.124	.111	.411	.522			
	101	.093	.022	26.940	.000			
	103	.052	.032	2.672	.102			
	105	.100	.192	17.630	.000			
	107	.339	.320	.436	.509			
	109	.098	.027	24.160	.000			
	111	.141	.148	.098	.754			
	113	.028	.020	.720	.396			
	115	.011	.020	1.480	.224			
	117	.004	.003	.065	.798			
D20S905						24.9	11	.009
	76	.003	.003	.001	.982			
	78	.038	.042	.080	.777			
	80	.008	.016	.937	.333			
	82	.014	.016	.063	.802			
	84	.264	.311	2.014	.156			
	86	.429	.376	2.187	.139			
	88	.223	.174	2.848	.092			
	90	.014	.053	9.457	.002			
	92	.000	.005	2.714	.099			
	94	.008	.000	4.268	.039			
	96	.000	.003	1.356	.244			
	98	.000	.003	1.356	.244			
D20S194						17.7	21	.67
	185	.003	.000	1.442	.230			
	187	.011	.003	2.105	.147			
	189	.011	.013	.065	.799			
	191	.003	.005	.290	.591			
	193	.025	.044	2.167	.141			
	195	.060	.065	.069	.793			
	197	.047	.047	.000	.991			
	199	.069	.063	.117	.733			
	201	.052	.076	1.707	.191			
	203	.088	.073	.569	.451			
	205	.146	.130	.373	.541			
	207	.107	.138	1.659	.198			
	209	.146	.117	1.325	.250			
	211	.060	.060	.001	.975			
	213	.096	.081	.553	.457			
	215	.025	.034	.549	.459			
	217	.016	.026	.826	.364			
	219	.019	.013	.458	.499			
	221	.008	.003	1.162	.281			
	223	.003	.003	.001	.970			
	225	.000	.005	2.672	.102			
	227	.005	.003	.397	.529			
D20S192						12.1	12	.44
	273	.000	.003	1.275	.259			
	275	.003	.000	1.507	.220			
	277	.012	.005	.939	.333			
	281	.015	.010	.261	.610			
	283	.003	.003	.007	.935			
	285	.436	.471	.929	.335			
	287	.020	.031	.838	.360			
	289	.088	.081	.115	.735			
	291	.114	.122	.121	.728			
	293	.298	.255	1.677	.195			
	295	.003	.016	3.436	.064			
	297	.006	.003	.468	.494			
	299	.003	.000	1.507	.220			
D20S156						3.7	5	.59
	181	.007	.000	1.097	.295			
	183	.149	.130	.297	.586			
	185	.518	.506	.050	.822			
	187	.298	.351	1.282	.257			
	189	.021	.013	.380	.538			
	191	.007	.000	1.097	.295			
D20S892						20.8	12	.05
	199	.006	.000	2.980	.084			
	201	.006	.000	2.980	.084			
	203	.106	.076	2.110	.146			
	205	.017	.036	2.622	.105			
	207	.011	.044	7.635	.006			
	209	.299	.284	.199	.656			
	211	.060	.047	.656	.418			
	213	.121	.130	.151	.698			
	215	.313	.307	.030	.863			
	217	.043	.057	.772	.380			
	219	.014	.013	.025	.876			
	221	.003	.003	.005	.944			
	223	.000	.003	1.292	.256			
D20S846						4.0	8	.85
	269	.349	.318	.832	.362			
	271	.009	.005	.300	.584			
	273	.261	.294	.991	.320			
	275	.071	.086	.565	.452			
	277	.017	.013	.202	.653			
	279	.153	.159	.041	.839			
	281	.102	.091	.261	.610			
	283	.034	.034	.000	.986			
	285	.003	.000	1.477	.224			

LRS, likelihood ratio statistics; DR, degrees of freedom.

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Letter to the Editors

Failure to find association between PRODH deletion and schizophrenia☆

Dear editors,

A deletion of the 22q11.2 region, which occurs in 1 of 4000 live births (Wilson et al., 1994), is a relatively common genetic disorder in humans. The 22q11.2 deletion has been found in 0.3–2% of adult patients with schizophrenia (Arinami et al., 2001; Karayiorgou et al., 1995) and in 6% of childhood-onset schizophrenia (Usiskin et al., 1999). It was reported that 24% of patients with VCFS met the criteria for schizophrenia (Murphy et al., 1999). Therefore, the risk of schizophrenia for patients with the 22q11.2 deletion may be 25–30 times higher than the general population risk of 1%. However, it is difficult to determine which of the specific genes in this genomic region may be associated with psychiatric problems.

The proline dehydrogenase gene (PRODH) is located in this region and has been suggested to increase susceptibility to schizophrenia because of abnormal findings in a PRODH-mutant mouse, allelic association with schizophrenia (Liu et al., 2002), and detection of a family with schizophrenia and a 350-kb deletion that includes PRODH (Jacquet et al., 2002). During our ongoing screening study for polymorphisms associated with schizophrenia in the 22q11.2 region, we identified one Japanese family with three members who carried the PRODH deletion. The father and two daughters of the family were hemizygous for at least a 100-kb region extending from single nucleotide polymorphism (SNP) rs416659 (dbSNP of National Center for Biotechnology Information (NCBI)) to D22S1638 because they each carried only one allele of the rs416659 and rs1210635 SNPs and D22S1638 and the father's allele was not transmitted to the daughters. The father's paternity was confirmed by testing of more than 400 microsatellite markers, which were used for a genome-wide linkage scan (Takahashi et al., 2003). The father's null allele should have been transmitted to the daughters.

To confirm the deletion, we devised a PCR-based homologous gene quantitative amplification screening method to detect the PRODH deletion. A primer set that amplifies an intronic region of PRODH and its counterpart, intronic region of ψPRODH was designed with the BLAST 2 (http://www.ncbi.nlm.nih.gov/blast/bl2seq/bl2.html). PCR was done with the following primers: forward, 5′-AGCTCAGTGCCCATGTCAGT and reverse, 5′-ACTGCCCTGTCTGCCTGTAG. The reverse primer was 5′-labeled with the fluorescein dye 6-FAM. The PCR product sizes from PRODH (NCBI accession number AC008103) and ψPRODH (AC007663) were 229 and 239 bp, respectively. After denaturation at 95 °C for 10 min, amplification consisted of 26 cycles of denaturation at 95 °C for 30 s, annealing at 62 °C for 30 s, and extension at 72 °C for 30 s followed by a final extension of 72 °C for 7 min. PCR product was mixed with ROX labeled GeneScan 400 HD. Electrophoresis was carried out with an ABI PRISM 3100 Genetic Analyzer (Applied Biosystems). Peak height of each PCR product from PRODH and ψPRODH was measured with the GeneScan and Genotyper programs (Applied Biosystems). The peak ratio of ψPRODH/PRODH was calculated. To monitor the quality of each experiment, samples from individuals with the PRODH deletion were amplified simultaneously.

The sequences of these regions are highly homologous, but the PCR product from PRODH was 10 bp shorter than that from ψPRODH (Fig. 1). In this

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