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Association of *SOX10* with schizophrenia in the Japanese population

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Background Microarray studies of schizophrenic brains revealed decreases in the expression of myelin and oligodendrocyte-related genes. Of these genes, sex-determining region Y-box 10 (*SOX10*) is a major transcription factor modulating the expression of proteins involved in neurogenesis and myelination. The *SOX10* gene is located on chromosome 22q13.1, a region repeatedly reported to show positive signals in linkage studies on schizophrenia.

Objective This study was conducted to clarify the exact role of *SOX10* in the pathophysiology of schizophrenia.

Methods We performed an association analysis of *SOX10* in a Japanese population of 915 schizophrenic patients and 927 controls. Genotyping was carried out using polymerase chain reaction restriction fragment length polymorphism.

Main results One single nucleotide polymorphism of the *SOX10* gene (rs139887) was selected as a haplotype tag single nucleotide polymorphism using 96 controls. A significant association was observed in the genotype and allelic frequency of this single nucleotide polymorphism between schizophrenic patients and controls ($P = 0.025$ and $P = 0.009$, respectively). Especially, a significant association was found in male patients, but not female patients. We also performed a mutational search of the whole coding region, branch site, and promoter region of *SOX10* in 96 schizophrenic patients, but no potential functional polymorphisms were detected.

Conclusion This study suggests that the *SOX10* gene is related to the development of schizophrenia in the Japanese population. *Psychiatr Genet* 17:227–231 © 2007 Lippincott Williams & Wilkins.

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Keywords: association study, myelin, oligodendrocyte, schizophrenia, single nucleotide polymorphism, *SOX10*

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Introduction

Functional abnormalities of neuronal connectivity have been reported in patients with schizophrenia (Liddle, 1996; McClure *et al.*, 1998). Histological, anatomical, and neuroimaging studies have led to the hypothesis that dysfunctional myelination during nerve fiber formation may contribute to the pathophysiology of schizophrenia (Davis *et al.*, 2003). Diffusion tensor imaging, which is considered to be a useful technique to estimate myelination *in vivo* (Klingberg *et al.*, 1999) showed decreased anisotropy in the prefrontal cortex, temporoparietal and parietooccipital regions, splenium, cingulum, and the posterior capsule and adjacent occipital white matter in schizophrenic patients compared with normal controls (Buchsbaum *et al.*, 1998; Lim *et al.*, 1999; Agartz *et al.*, 2001). In addition, several postmortem studies have demonstrated reductions in glial cell density (Rajkowska *et al.*, 1999) and neuronal size in the cerebral cortex of affected patients (Rajkowska *et al.*, 1999; Pierri *et al.*, 2001; Chana *et al.*, 2003). Thus, schizophrenic brain changes may occur not only in gray but also in white matter.

Recently, several groups identified decreased expression of myelin and oligodendrocyte-related genes in patients with schizophrenia or bipolar disorder using gene microarrays (Hakak *et al.*, 2001; Tkachev *et al.*, 2003; Dracheva *et al.*, 2006). Of these genes, the expression of sex-determining region Y-box 10 (*SOX10*), a major transcription-modulating factor involved in neurogenesis and myelination in the central and peripheral nervous systems (Kuhlbrodt *et al.*, 1998; Paratore *et al.*, 2001; Potterf *et al.*, 2001) was significantly decreased. In addition, dysfunction of *SOX10* causes central dysmyelinating leukodystrophy

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(Pingault *et al.*, 1998), and up to 53% of patients with leukodystrophy concomitantly were reported to develop psychotic symptoms in adolescence and young adulthood (Hyde *et al.*, 1992).

The gene-encoding *SOX*10 is located on chromosome 22q13.1; this locus includes a susceptibility gene locus linked to schizophrenia (Mowry *et al.*, 2004), which contains several candidate genes such as apolipoprotein L1 (*APOL1*) (Mimmack *et al.*, 2002), cytochrome P450 family 2 subfamily D polypeptide 6 (*CYP2D6*) (Fu *et al.*, 2006), phospholipase A2, group VI (*PLA2G6*) (Yu *et al.*, 2005), and protein interacting with PRKCA1 (*PICK1*) (Hong *et al.*, 2004).

Taken together, these data suggest that *SOX*10 is a plausible candidate gene playing a role in the pathophysiology of schizophrenia, but the exact role of *SOX*10 in the development of schizophrenia remains to be clarified. In this study, we performed a linkage disequilibrium (LD) analysis of *SOX*10, followed by case–control studies examining the association between *SOX*10 polymorphism and schizophrenia using a large population.

Patients and methods

Patients

The participants consisted of 915 patients with schizophrenia (398 females: mean age ± standard deviation 50.1 ± 16.2; 517 males: 47.4 ± 14.8) and 927 controls (472 females: 38.5 ± 14.7; 455 males: 38.0 ± 14.3 years).

LD was evaluated using 96 control participants, whereas mutation screening was performed using 96 schizophrenic patients. Both of these subsets were randomly selected from the present participants. All participants were unrelated to each other and ethnically Japanese. A consensus diagnosis based on unstructured interviews was made for each patient by experienced psychiatrists according to the *Diagnostic and statistical manual of mental disorders*, 4th ed. (DSM-IV; American Psychiatric Association, 1994). All available medical records and information from family members were also taken into consideration. Mentally healthy controls had no current or past contact with psychiatric services based on unstructured interviews. This study was approved by the Ethics Committee of Nagoya University Graduate School of Medicine and Fujita Health University.

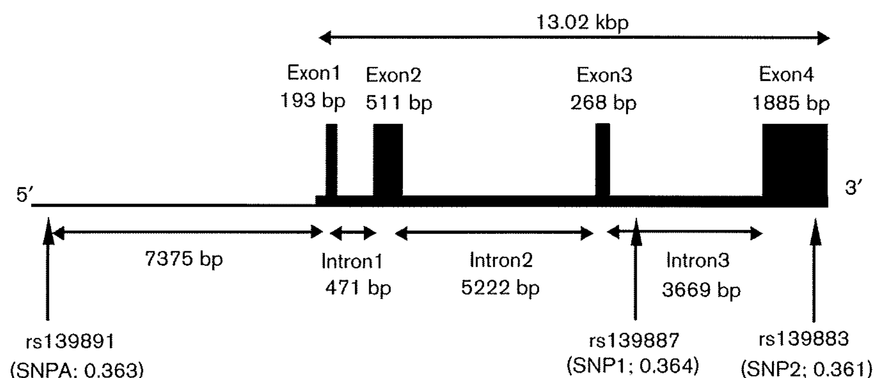
Genotyping

The *SOX*10 gene is composed of four exons, spanning a total length of 13.02 kb. According to the information on *SOX*10 genetic single nucleotide polymorphisms (SNPs) provided by the dbSNP database (*http://www.ncbi.nlm.nih.gov/SNP/*) we selected three SNPs, of which the minor allele frequencies are higher than 20%, for LD mapping analysis: SNPA (rs1398891 is 7375-bp upstream of *SOX*10), SNP1 (rs139887), and SNP2 (rs139883) (Fig. 1). Genomic DNA was extracted from the peripheral blood of all participants. Genotyping was performed by PCR restriction fragment length polymorphism (PCR-RFLP). Each primer and restriction enzyme used for the genotyping of SNPA, SNP1, and SNP2 by PCR-RFLP is described in Table 1.

Mutation search

Primer pairs were designed using information from GenBank sequence (accession number: NT_011520.10). Mutation search was performed in the whole coding regions, the promoter regions, the branch site, and 5′-flanking regions (500-bp upstream from the initial exons of the gene). Direct sequencing was performed using an ABI PRISM 3100 Genetic Analyzer (Applied Biosystems Tokyo, Japan Ltd).

Fig. 1



Genomic structure of *SOX*10 with single nucleotide polymorphisms (SNPs) in linkage disequilibrium mapping. Numbers under the arrows represent SNP IDs. Parenthetic numbers represent the three SNPs that we selected and the minor allele frequencies (MAFs) of 96 controls.

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Statistical analysis

Genotype deviation from the Hardy–Weinberg equilibrium (HWE) expectation was evaluated using the χ^2 test. The association between patients with schizophrenia and controls for genotype and allele distributions were also evaluated by χ^2 test. To evaluate pairwise LD matrices among SNPs (by D′ and r^2), we used HAPLOVIEW version 3.0 software (developed by Mark Daly; *http://www.broad.mit.edu/personal/jcbarret/haploview/index.php*). Haplotype tag SNPs (htSNPs) were then selected using the same program, as described by Johnson *et al.* (2001). The significance level for all statistical tests was set at 0.05.

Results

The genotype frequencies for all SNPs examined were in HWE, and all the D' values between the possible combinations of SNPs were 1.0. The r^2 values for SNPA–SNP1, SNPA–SNP2, and SNP1–SNP2 were 0.93, 0.96, and 0.95, respectively. As the *SOX10* genes are considered a single strong LD block, SNP1 was selected as an htSNP for this block.

The genotype distributions and allele frequencies of SNP1 in schizophrenic patients and controls are summarized in Table 2. The genotype frequency of SNP1 was not significantly different from the distribution expected from the HWE in 915 Japanese schizophrenic patients and 927 Japanese controls. The minor allele frequency decreased significantly in schizophrenic patients from that seen in normal controls. Association analysis revealed a significant difference in SNP1 genotype and allele distribution between controls and patients with schizophrenia ($P = 0.025$ and $P = 0.009$, respectively). Moreover, in view of the sex difference in gene effects, we included analyses of samples divided accordingly. As a result, significant association was found in SNP1 genotype and allele distribution between controls and male schizophrenic patients ($P = 0.010$ and $P = 0.006$, respectively), whereas no association was found in female patients.

No polymorphisms gave rise to nonsynonymous changes in the mutation search of 96 schizophrenic patients.

Discussion

In this study, we observed a significant difference in the minor allele frequency of SNP1 between schizophrenic patients and healthy controls. Our results suggest that the *SOX10* gene is related to the development of schizophrenia in the Japanese population, which may support the findings of microarray studies (Hakak *et al.*, 2001; Tkachev *et al.*, 2003) showing a significant decrease in the expression of the *SOX10* gene in the prefrontal cortex of postmortem brains in patients with schizophrenia.

Genetic variations in *SOX10* may influence the formation and maintenance of myelin sheath and may give rise to dysfunctional synaptic connectivity. These variations may be responsible for an individual patient's vulnerability for

Table 1 Primer and restriction enzymes used for genotyping

SNP	PCR primer sequence (5′–3′)	Product size (bp)	Annealing temperature	Restriction enzyme	Variants
rs139891 (SNPA)					
Forward	TTCCCATGCCTTCAGAGTTC	156	64	StyI	T/C
Reverse	TGCCTGCACACATTCTCTTC				
rs139887 (SNP1)					
Forward	GGGAGACCAGAGGAGGAGTC	180	64	HinfI	C/G
Reverse	CAGGGACACACACACACACA				
rs139883 (SNP2)					
Forward	TCCACTAAGTCCCTCGAACC	203	64	MspI	T/C
Reverse	GGAGGCCTTACCACTCCTATG				

Table 2 Genotypic analysis of SNP1 in SOX10 gene

	Number	Genotype			P value (genotype)	MAF	P value (allele)	OR	95% CI
		C/C	G/C	G/G					
Total									
Schizophrenia	915	456 (50%)	385 (42%)	74 (8%)	0.025	0.29	0.009	0.83	0.72–0.95
Control	927	405 (44%)	430 (46%)	92 (10%)		0.33			
Female									
Schizophrenia	398	192 (48%)	174 (44%)	32 (8%)	0.621	0.30	0.377	0.91	0.74–1.12
Control	472	217 (46%)	209 (44%)	46 (10%)		0.32			
Male									
Schizophrenia	517	264 (51%)	211 (41%)	42 (8%)	0.010	0.29	0.006	0.76	0.63–0.92
Control	455	188 (41%)	221 (49%)	46 (10%)		0.34			

CI, confidence interval; MAP, minor allele frequency; OR, odds ratio.

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the development of schizophrenia. Oligodendrocytes function in the formation of myelin, express neurotransmitter receptors and facilitate interactions between neurons and glia during synaptic transmission (Haydon, 2001), and changes in *SOX*10 activity may cause alterations in neurotransmitter-triggered signal transduction and subsequent neuronal activity. Moreover, stratified by sex, only male patients showed significant association. This result could stem from different mechanism of myelination between sexes (Chambers and Perrone-Bizzozero, 2004). This speculation, however, needs further confirmation.

The SNP significantly associated with schizophrenia in this study is located within intron three of *SOX*10. As it is located outside the region affecting mRNA splicing, it seems unlikely to influence *SOX*10 function directly. An alternative possible interpretation is that the functional variant in LD with the minor allele of this SNP may work protectively against schizophrenia in controls. Considering that an actual susceptible polymorphism to schizophrenia may be located within the LD of this SNP, we examined a total of 96 patients with schizophrenia in this mutational search to detect a variant present at more than 5% with 95% power (Collins and Schwartz, 2002). No potentially functional polymorphism with a significant frequency was, however, found in this population.

The LD pattern observed in our sample was almost the same as that provided by the HapMap Project (HapMap data Release Number 16c.1: *http://www.hapmap.org/*). The block examined here covers the entirety of the *SOX*10 gene; however, the gene is included in a larger LD block according to the SNP browser data (SNP browser software version 3.0: *http://www.allsnps.com/snpbrowser*). Therefore, SNP1 may be in LD with an actual schizophrenia-susceptibility SNP located within another gene or noncoding DNA that can regulate gene transcription (Carninci *et al.*, 2005). To examine this possibility further, a complete mutational search of the genes surrounding *SOX*10 is needed. For example, polymerase (RNA) II (DNA-directed) polypeptide F (*POLR2F*), which is approximately 4 kbp downstream of *SOX*10, or protein interacting with PRKCA1 (*PICK1*), which is approximately 70 kbp upstream of *SOX*10, could be appropriate for these studies.

Several limitations could be considered in this study. First, there was a substantial demographic difference between the two groups and this may affect the present results. Second, the Japanese population is considered to be ethnically homogeneous; however, unidentified sample stratification could exist in this cohort, and we did not test for such stratification. Therefore, the undetectable results in our mutational search may reflect such latent sample stratification.

Recently, patients with schizophrenia were reported to exhibit increased *SOX*10 methylation, leading to reduced expression compared with control participants (Iwamoto *et al.*, 2005). Additionally, a striking contrast to our results, Iwamoto *et al.* (2006) reported that the genetic variations in the *SOX*10 gene do not contribute to susceptibility to Japanese schizophrenia. Further research is needed to elucidate the exact relationship between functional changes in the effects of *SOX*10 gene owing to the genetic variations and the pathophysiology of schizophrenia. Although our sample size is relatively large, replication of this study using independent sample sets will be required to confirm our findings.

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Association study between the *transferrin* gene and schizophrenia in the Japanese population

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Several lines of evidence, including diffusion tensor imaging and microarray studies, indicate that abnormalities in myelination play an important role in the pathophysiology of schizophrenia. Of myelin and oligodendrocyte-related genes, a significant decrease in the mRNA levels of transferrin in schizophrenics has been reported by both microarray and quantitative polymerase chain reaction studies. We performed an association analysis of the transferrin gene in a Japanese population of 384 schizophrenic patients and 384 controls. Six single nucleotide polymorphisms were genotyped by polymerase chain reaction-restriction fragment length polymorphism and a TaqMan assay. No significant differences in genotype, allele, or haplotype frequencies of the six single nucleotide polymorphisms were observed between schizophrenic patients and controls. The present results suggest that the *transferrin* gene is not related to the development of schizophrenia in the Japanese population. *NeuroReport* 18:517–520 © 2007 Lippincott Williams & Wilkins.

Keywords: association study, myelin, oligodendrocyte, schizophrenia, single nucleotide polymorphism, transferrin gene

Introduction

Numerous lines of evidence have suggested that disturbances in myelination contribute to the pathophysiology of schizophrenia. Several postmortem brain studies have demonstrated a reduction in glial cell density in affected patients [1,2]. In addition, diffusion tensor imaging (DTI), which is a useful technique for estimating myelination *in vivo* [3], showed decreased anisotropy in various regions of white matter in schizophrenic patients relative to normal controls. These regions included the prefrontal cortex, splenium, and cingulum, all of which are considered related to the pathophysiology of schizophrenia [4,5]. More recently, several groups reported decreased expression of myelin and oligodendrocyte-related genes in patients with schizophrenia using gene microarrays and quantitative polymerase chain reaction analysis [6,7]. In these studies, a highly significant decrease in transferrin expression was reported.

Transferrin is an iron transport glycoprotein synthesized primarily by hepatocytes; it is also synthesized by oligodendrocytes. Several lines of evidence indicate that brain transferrin could be involved in myelinogenesis [8]. For example, a selective culture that is essential for cultivation of neural stem cells (called the neurosphere method) contains transferrin and actually favors the outgrowth of the neurosphere [9].

Moreover, increased mRNA levels of myelin and oligodendrocyte-related genes, including *PLP, MBP, CNP, MAG, OLIG2,* and *SOX10,* have been reported in a study of transgenic mice overexpressing the complete human *transferrin* gene (*TF*) in oligodendrocytes [10]. These results suggest that transferrin can influence the maturation and differentiation of oligodendrocyte in the central nervous system [10].

The gene encoding transferrin is located on chromosome 3q22.1; linkage analyses using a genome-wide map of microsatellite DNA markers showed that the region of chromosomal 3q is implicated in the susceptibility loci for schizophrenia [11–13].

Additionally, the concentration of transferrin changes during the acute phase of schizophrenia [14,15]. Furthermore, Wong *et al.* reported that transferrin levels are reduced in schizophrenic patients relative to normal controls and can be affected by medications and disease stages [15].

Taken together, *TF* is considered a plausible candidate gene for a role in the pathophysiology of schizophrenia, although its exact role in the development of schizophrenia remains to be clarified.

Thus, in this study, we performed linkage disequilibrium (LD) analysis of *TF* followed by case–control studies examining the possible association between *transferrin* gene polymorphisms and schizophrenia in a Japanese population.

Participants and methods

Participants

The participants were 384 patients with schizophrenia [165 women, mean age ± standard deviation (SD) 56.4 ± 14.3; 219

men, 52.6±12.8] and 384 controls (162 women, 46.0±18.0; 222 men, 44.2±15.2 years).

All participants were unrelated and ethnically Japanese. A consensus diagnosis was made for each patient by experienced psychiatrists according to the *Diagnostic and Statistical Manual of Mental Disorders*, 4th ed. (American Psychiatric Association, 1994), and all available medical records and information from family members were also taken into consideration. Mentally healthy controls had no current or past contact with psychiatric services, according to unstructured interviews. This study was approved by the Ethics Committee of the Nagoya University School of Medicine, and written informed consent was obtained from all participants.

Single nucleotide polymorphism selection

TF is composed of 17 exons spanning a total length of 32.4 kb. Only one isoform of *TF* has been registered in NCBI GenBank (NM_001063_2). According to the information regarding single nucleotide polymorphisms (SNPs) of *TF* found in the dbSNP database (*http://www.ncbi.nlm.nih.gov/SNP/*) and the HapMap database (*http://www.hapmap.org/index.html.ja*: Release #19/ phase II October, 2005), the LD block was determined on the basis of the criterion $D' > 0.80$ using HAPLOVIEW software ver. 3.2 (developed by Mark Daly, URL: *http://www.broad.mit.edu/personal/jcbarret/haploview/index.php*; [16]). Haplotype tag SNPs (htSNPs) were defined as those capturing 90% of the haplotype diversity within each of the LD blocks using the same program. *TF* was constructed from a single LD block, and five SNPs (SNP1: rs8177191, SNP4: rs1799852, SNP9: rs3811647, SNP10: rs1358024, and SNP12: rs8649) were selected as htSNPs. A nonsynonymous variant SNP (SNP14: rs1049296) of *TF* was included in the analyses (Figs 1 and 2).

Single nucleotide polymorphism genotyping

Genomic DNA was extracted from the peripheral blood of all participants. rs817791 was genotyped by polymerase chain reaction-restriction fragment length polymorphism using primers (left: 5′-CCTTCTTTGGCAGCTTTTGA-3′, right: 5′-CCCTGAGGGTAAGGACCAAT-3′) and the restriction enzyme PvuII. Genotyping of the other SNPs was carried out using TaqMan assays (Applied Biosystems, Foster City, Calfornia, USA). TaqMan probes and Universal PCR Master Mix were obtained from Applied Biosystems. A 5 μl total reaction volume was used and allelic-specific

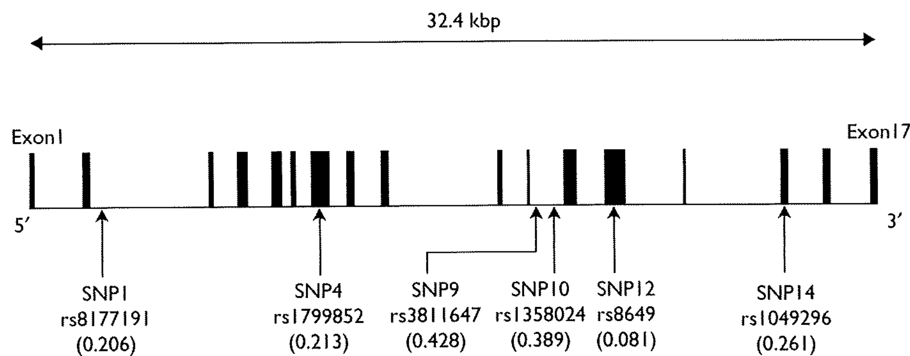

Fig. 1 Genomic structure of *TF* with single nucleotide polymorphisms (SNPs) in linkage disequilibrium mapping. Numbers under the arrows represent the six SNPs that we selected and SNPs IDs. Parenthetic numbers represent the minor allele frequencies of 384 controls.

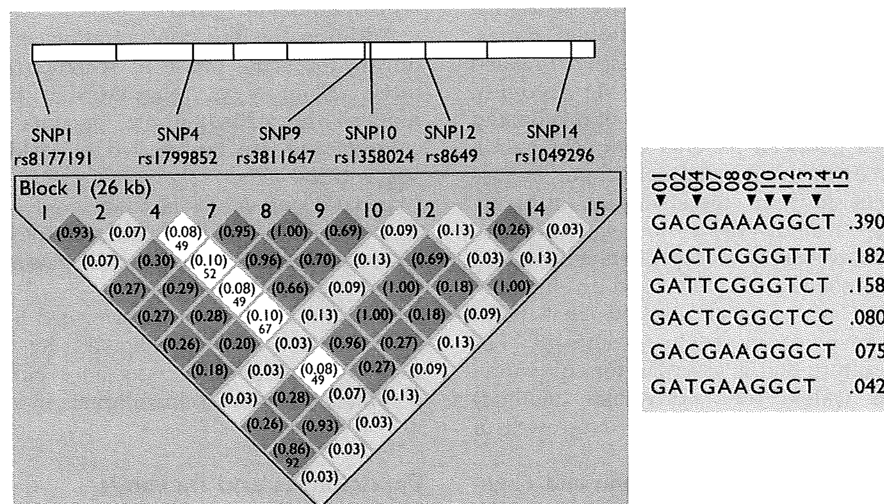

Fig. 2 (a) Linkage disequilibrium of *TF* provided by Hapmap database (release #19/ phase II October, 2005). Numbers in the box represent D' values (D' values of 1.0 are not shown). Parenthetic numbers represent the r^2 value. (b) Figure represents a pair of haplotype frequencies in *TF*.

fluorescence was measured using an ABI PRISM 7900 Sequence Detector System (Applied Biosystems).

Statistical analysis

Genotype deviation from Hardy–Weinberg equilibrium was evaluated using a χ^2 test. The associations between patients or controls with genotype and allele distributions were also evaluated using a χ^2 test. Haplotype analyses were performed using COCAPHASE version 2.403 (*http://portal.-litbio.org/Registered/Option/unphased*; Dudbridge, 2003). The significance level for all statistical tests was set to 0.05. Power calculations were performed using the Genetic Power Calculator genetic statistics package (*http://pngu.mgh.harvard.edu/~purcell/gpc/*; Purcell 2001–2005).

Results

The observed genotype frequencies of the six SNPs were within the distributions expected by Hardy–Weinberg equilibrium. The LD patterns observed in our patients and controls were nearly identical to those provided by the HapMap Project.

The genotype distributions and allele frequencies of the six SNPs in the Japanese schizophrenic patients and controls are summarized in Table 1. No significant differences in genotype and allele frequencies of five htSNPs and the nonsynonymous variant SNP were observed between schizophrenic patients and controls. The distribution of haplotypic frequencies of SNP1–SNP4 (G-C) differed between the schizophrenic patients and controls (individual haplotypes, $P=0.03918$), but this association disappeared after correcting for multiple testing by a 10 000-iteration permutation test ($P=0.1703$) (Table 1).

Discussion

This study suggests that *TF* does not play an important role in the development of schizophrenia in the Japanese population, as our association analysis failed to reveal a significant association between polymorphisms of the gene and schizophrenia. As it is, however, expected that genetic risk factors for schizophrenia may differ between races or ethnicities, a replication study using different ethnic populations would be required to confirm our results.

Although highly significant decreases in the expression of *TF* in postmortem brains of schizophrenic patients have been reported, a recent study revealed that mRNA levels of brain *TF* are strongly regulated by the mRNA levels of quaking homolog, KH domain RNA binding (*QKI*) in the brains of schizophrenic patients [17]. QKI is an RNA-binding protein that regulates splicing processes subsequent to transcription [18], and is thought to regulate oligodendrocyte differentiation and maturation in the human brain [19]. It would therefore be of value to further investigate the gene–gene interactions between *TF* and *QKI* during the development of schizophrenia.

Although the levels of transferrin receptor (TfR) in the central nervous system have not been clarified, the elevation of plasma TfR in schizophrenia has been reported [20,21], and it could be hypothesized that the decrease in *TF* mRNA levels in postmortem brains observed in previous studies might reflect changes in TfR rather than sequence variations in *TF*. Therefore, it would be useful to investigate the genetic association of the *TfR* gene (*TFRC*) with schizophrenia.

Table 1 Association analyses of six SNPs in transferrin gene

SNP ID	Genotypic distribution[a]							Allelic distribution			Multi-haplotype systems[b]					
	M/M		M/m		m/m		P values (genotype)	GRR	MAF (%)		1SNP	2SNP	3SNP	4SNP	5SNP	6SNP
	SCZ	CON	SCZ	CON	SCZ	CON			SCZ	CON						
SNP 1(G/A) (rs8177191)	248	243	122	121	12	18	0.534	1.4	19.1	20.6	0.48925					
												0.0363				
SNP 4(C/T) (rs1799852)	263	235	105	131	16	16	0.109	1.4	17.8	21.3	0.08449		0.19535			
												0.2126		0.19542		
SNP 9(G/A) (rs3811647)	105	129	186	180	82	74	0.242	1.33	46.9	42.8	0.1092		0.2049		0.33119	
												0.05598		0.2053		0.31334
SNP 10(G/A) (rs1358024)	128	144	195	180	61	59	0.455	1.34	41.3	38.9	0.3431		0.0527		0.1377	
												0.392		0.2003		
SNP 12(G/C) (rs8649)	317	325	62	56	4	3	0.761	1.61	9.1	8.1	0.4567		0.3907			
												0.2657				
SNP 14(C/T) (rs1049296)	225	214	141	138	16	31	0.078	1.37	22.6	26.1	0.1143					
Permutation *P* value[c]												0.1703				

[a]CON, control; GRR, genotype relative risk; M, major allele; m, minor allele; MAF, minor allele frequency; SCZ, schizophrenia; SNP, single nucleotide polymorphism.
[b]*P* values were calculated by log-likelihood ratio test (1SNP; allelewise association, 2–6SNP; global haplotypic association).
[c]An implement in the COCAPHASE.
Bold numbers represent significant *P* values.

Furthermore, polymorphisms in *TF* are significantly associated with the risk of Alzheimer's disease [22,23]. As alteration of cognitive ability is considered a fundamental dysfunction in both Alzheimer's disease and schizophrenia, association analysis using participants and information regarding their cognitive abilities might help elucidate the pathophysiology of schizophrenia.

Several caveats should be considered when interpreting our results. First, we obtained more than 80% of power to detect possible associations of the studied SNPs, except SNP12, when we set the GRR at 1.33–1.40. For SNP12, however, we could not eliminate the possibility of the type II error owing to the small sample size. Further studies using more samples are therefore needed. Second, we selected htSNPs so as to cover 90% of the haplotypes within each LD block, but because the LD block structure of *TF* was not tight in our control samples or the HapMap database (e.g. the r^2 values between SNP4 and the others were less than 0.8), it is possible that the htSNPs used in this study did not capture all haplotypes in the gene. In other words, there may exist SNPs not found in the LD for which we did not investigate the possible association with schizophrenia. Hence, further analyses on the basis of more comprehensive and detailed SNP coverage of this gene are necessary to make conclusive results.

Conclusion

We concluded that *TF* does not play an important role in the development of schizophrenia in the Japanese population.

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Regular Article

Age of onset has limited association with body mass index at time of presentation for anorexia nervosa: Comparison of peak-onset and late-onset anorexia nervosa groups

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Abstract The clinical characteristics differentiating late-onset anorexia nervosa (AN) from typical pubertal onset AN remain unclear. The purpose of the present study was to examine these differences in a retrospective analysis. A total of 149 female AN patients was divided into two groups: a peak-onset AN group ($n = 125$) in which onset occurred between the ages of 15 and 24 years, and a late-onset AN group ($n = 24$) in which onset occurred at the age of ≥25 years. A logistic regression analysis was conducted with this classification as the target variable and five clinical factors as explanatory variables for the clinical characteristics at the time of initial examination. Body mass index (BMI) at the time of presentation was identified as a possible factor affecting classification as peak-onset or late-onset AN. In addition, a negative linear correlation was detected between age of onset and BMI at the time of initial examination. The results suggest that BMI at the time of the initial examination is an important clinical characteristic to differentiate peak-onset AN and late-onset AN.

Key words age of onset, anorexia nervosa, body mass index, eating disorders, retrospective studies.

INTRODUCTION

Since the time Morton first described eating disorders as a medical condition in the late 17th century,[1] puberty has been considered the most common period for the onset of these disorders. For example, in the diagnostic criteria for eating disorders produced by Feighner *et al.* in 1972, age at onset <25 years was established as a diagnostic criterion.[2] Therefore, the pathology of eating disorders has been hypothesized from the biological, physiological, and social changes that occur in puberty. However, the existence of late-onset anorexia nervosa (AN) has been documented.[3] For example, case reports on late-onset AN have been seen sporadically since the 1970s,[4–8] and compared with AN occurring in puberty (peak-onset AN).

Some authors have noted that late-onset AN is related to various separation experiences that occur in middle and old age (e.g. death of a spouse or independence of children). From an investigation of 50 cases of anorexia tardive with patients ranging in age from 21 to 80 years, Dally described a relationship between this condition and marriage, birth, and spouses, and emphasized its difference to peak-onset AN.[9]

Further findings on peak-onset and late-onset AN are described in the following. A very similar clinical picture of peak-onset AN and late-onset AN was reported in terms of time from onset until consultation with a clinician, weight at time of onset, maximum weight, minimum weight, and frequency of overeating

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and vomiting.[10] In contrast another comparative study found that late-onset AN patients had lower weight at the time of initial examination than peak-onset AN patients.[11]

Other comparisons have indicated that late-onset AN patients have a worse prognosis than peak-onset patients.[3,12] However, there is currently no consensus of opinion on the differences in clinical characteristics between peak-onset and late-onset AN.

The authors reported a patient with extremely low bodyweight (body mass index [BMI]: 10.8 kg/m^2) at the time of presentation, and focused on mid-life crisis in four patients diagnosed with late-onset AN.[13] This led us to question the general differences in clinical characteristics between peak-onset and late-onset AN at the time of initial examination.

Steinhausen reviewed the factors that are generally viewed to be clinical characteristics of eating disorders, and identified age at onset, period until treatment, period of treatment in hospital, amount of weight loss, vomiting, overeating and overuse of purgatives, developmental problems, parent–child relationship, chronicity, hysteric personality, and compulsive personality as factors affecting course and outcome.[14]

Thus, in the present study we conducted a retrospective comparison of the available clinical characteristics at the time of initial examination in cases of peak-onset and late-onset AN.

METHODS

Four hundred women who were initially examined in the psychiatry department of Nagoya Daini Red Cross Hospital between 1 January 1998 and 31 December 2003, and who fulfilled the criteria for eating disorders on the DSM-IV,[15] were potential subjects for the study. Four hundred of 406 patients identified during that period as having eating disorders were female. Because the overwhelming majority of such patients were female, the subjects of the present analysis were limited to women. After eliminating 251 bulimia nervosa patients, the remaining 149 AN patients were divided into two groups based on age of onset, and a comparison was conducted of these two groups. Although some studies had used an age of onset of >30 years for the diagnosis of late-onset eating disorders, in the present study the age of 25 years was adopted, the same as that used by Feighner *et al.*,[2] Boast *et al.*,[11] and others, and the subjects were divided into two groups: peak-onset AN (onset between 15 and 24 years), and late-onset AN (onset at age ≥25). Of the 149 female AN patients, 125 (age 15–24 years, mean age 17.8 ± 2.5 years) had peak-onset AN, and 24 (age 25–50 years, mean age 29.9 ± 5.9) had late-onset AN.

Patient information was obtained at the time of initial examination in the psychiatry department. Height and weight were not obtained by interview but by measurement on a bodyweight and height scale. BMI was calculated from a measurement of the height and weight. The difference between maximum and minimum BMI was obtained at the time of initial examination by asking the patient (and/or family member) her maximum and minimum bodyweight up to that time. The diagnosis and assessment at initial examination were made through a patient interview by a psychiatrist experienced in treating eating disorders. Although subclinical states on adolescence of late-onset AN was evaluated carefully, there is a limitation due to the hidden medical history on eating disorders.

According to factors suggested by Steinhausen, five factors including BMI, period from onset until the initial examination, subtypes of binge-eating/purging or restricting, difference between maximum and minimum BMI and birth order[14] were extracted from medical records for the present study. Duration of illness was investigated by detailed clinical interviews with patients and their parents. The onset of illness was defined when patients first met the AN criteria in DSM-IV. The binge-eating/purging type and restricting type were diagnosed with DSM-IV criteria. Difference between maximum and minimum BMI was taken from the maximum BMI until the initial examination and minimum BMI during illness.

The present study was approved under the guideline for epidemiological studies by the Ethical Committee of Nagoya University Graduate School of Medicine.

Nagoya Daini Red Cross Hospital is located in west central Nagoya (population approx. 2.1 million). The hospital plays a central role in the community with a focus on emergency medical care. In the area of eating disorders, the hospital's psychiatry department conducts outpatient treatment with a focus on psychological education, hospital treatment for low bodyweight,[16] and psychological interviews by a clinical psychotherapist. The hospital functions as a treatment center for eating disorders in the city of Nagoya.

Statistical analysis

The dependent variable was the classification of the two groups of peak-onset and late-onset AN. A logistic regression analysis was then carried out with five probable independent variables of BMI at the time of initial examination; period from onset until the initial examination; subtypes of binge-eating/purging or restricting; difference between maximum and minimum BMI; and birth order. All statistical analysis was done with SPSS for Windows version 11.5 (SPSS Japan, Tokyo, Japan).

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Table 1. Comparison of five factors in peak-onset and late-onset AN

	Peak-onset AN	Late-onset AN
No. patients	125	24
Mean age (years)	17.8 ± 2.5	29.9 ± 5.9
Birth order		
First	54	12
Others	71	12
Restricting type	109	21
Binge-eating/purging type	16	3
BMI (max–min)	6.7 ± 2.5	7.2 ± 2.8
BMI (initial exam)	13.4 ± 1.9	12.6 ± 2.0
Period (years)	2.8 ± 3.7	2.2 ± 2.0

AN, anorexia nervosa; BMI, body mass index; BMI (max–min), difference between maximum BMI until the initial examination and minimum BMI during illness; BMI (initial exam), BMI at the time of initial examination; period, periods from onset until the time of initial examination.

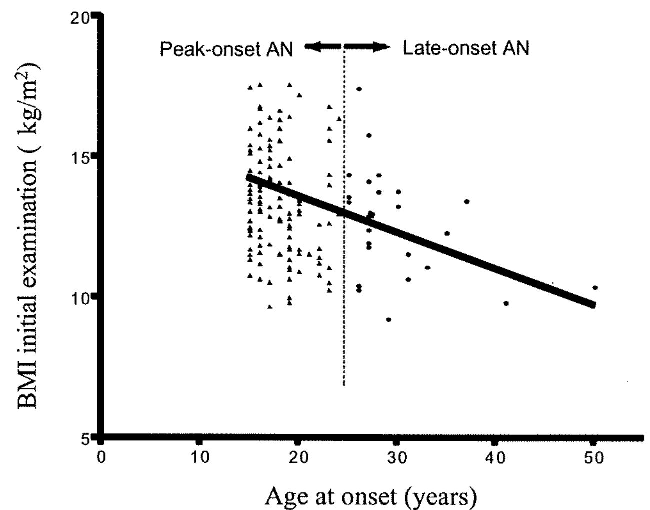

Figure 1. Relationship between age at onset and body mass index (BMI) at the time of initial examination. A significant linear correlation was seen ($y = 15.6 - 0.12x$; $r^2 = 0.064$; $P = 0.0018$). (▲) Peak-onset anorexia nervosa (AN); (●) late-onset AN.

RESULTS

The logistic regression analysis including five factors showed that BMI at the time of initial examination had the largest impact on classification as peak-onset AN or late-onset AN (odds ratio 1.26, $P = 0.052$). The other four factors were not significantly associated with their classification (period from onset until the initial examination: odds ratio 0.90, $P = 0.27$; subtype of binge-eating/purging or restricting: odds ratio 1.09, $P = 0.89$; difference between maximum and minimum BMI: odds ratio 0.93, $P = 0.51$; birth order: odds ratio 0.82, $P = 0.67$). BMI at the time of initial examination was close to being significantly lower in the late-onset AN group than in the peak-onset AN group (Table 1).

Because logistic regression indicated that BMI at the time of initial examination was a factor demarcating the two groups of peak-onset AN and late-onset AN, it was important to analyze the correlation between the age of onset and BMI at the time of initial examination for all patients. BMI was investigated with age at onset in a simple regression analysis. A significant correlation was seen between age at onset and BMI at the time of initial examination ($r^2 = 0.064$, $P = 0.0018$), but the contribution of age at onset to BMI at the time of initial examination was approximately 6.0%. (Common regression formula: $y = 15.6 - 0.12x$; $r^2 = 0.064$), where y is BMI at the time of initial examination and x is age at onset.) The number of patients with age at onset in the 40s and 50s was only one for each, and their BMI was considerably low among all patients; therefore the data for these two patients were excluded. Even though these two patients were excluded there was still a significant correlation ($P = 0.026$; Fig. 1).

DISCUSSION

The present study showed that BMI at the time of the initial examination had the largest influence on classification for peak-onset AN and late-onset AN among the five factors (BMI at the time of initial examination, period from onset until the initial examination, subtypes of binge-eating/purging or restricting, difference between maximum and minimum BMI, and birth order). Moreover, BMI decreased as age of onset increased. Thus, BMI at the time of the initial examination is an important clinical characteristic to differentiate peak-onset AN and late-onset AN.

The late-onset AN patients might not be able to face their painful intrapsychic anxiety in adolescence, and they might be in false adaptation by formulating a severe pathological denial for a long time. When it becomes impossible to maintain their denial they must suddenly face their accumulated anxiety. This situation might induce more rapid and larger BMI reduction in late-onset AN patients.

Boast *et al.* studied 186 AN patients including a peak-onset AN group with onset 15–19 years of age, and a late-onset AN group with onset >25 years.[11] Their results were similar to the present findings, with the late-onset group having a lower bodyweight than the peak-onset group at presentation. According to several

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follow-up studies, the clinical significance of BMI at presentation has been shown to be a predictive factor for a poor outcome.[17–19] Therefore, the low BMI at presentation in the current late-onset AN patients suggests the possibility that late-onset AN has a poorer prognosis than peak-onset AN, although this possibility needs to be investigated in future studies.

Joughin *et al.*, in contrast, compared 427 AN patients consisting of an early onset group in which onset was at 15–19 years of age, and a late-onset group in which onset was after the age of 30, and reported a very similar clinical picture between the two groups.[10] In their study the age of division between peak-onset and late-onset AN was different to the present one. In addition they used bodyweight rather than BMI at the time of initial examination, although simple bodyweight has a different meaning than BMI, which takes into account the factor of height.

Although the present study found no significant differences between the late-onset AN and peak-onset AN groups with regard to period from onset until the initial examination, binge-eating/purging type or restricting type, difference between maximum and minimum BMI, and birth order, some concerns should be taken into account.

First, Beck *et al.* reported that the time from onset to presentation of late-onset AN is longer than that for peak-onset AN due to poor insight into the disease.[20] It has been reported that AN is not readily noticeable for a long period of time when the onset occurs after marriage.[21] However, these studies lacked direct comparisons of peak-onset AN and late-onset AN groups. The present study involved both peak-onset and late-onset AN patients, and therefore had an advantage in being able to compare them within the study.

Second, recent studies have indicated no difference in terms of birth order in relation to AN,[22] although several studies showed that AN is more prevalent among firstborns[23] while others found that it is more prevalent in children born second or later.[24] Regarding the relation between birth order and age of onset of AN, no difference was found even in a study that compared AN with onset before the age of 14 and AN with onset after that age.[25] The present results provide further evidence that there is no significant difference in birth order between peak-onset AN and late-onset AN.

Interestingly, clinical features of late-onset AN are similar to those of peak-onset AN except for BMI at the time of initial examination. This may be related to the common psychopathology of late-onset AN and peak-onset AN.

In conclusion, BMI at the time of initial examination is an important clinical characteristic to differentiate peak-onset AN and late-onset AN. This was a retrospective study and was unable to adequately investigate outcome or to consider factors such as personality traits, early experience, and social support, which remain to be investigated.

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Successful Treatment of Trigeminal Neuralgia With Milnacipran

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Abstract

Paroxysmal pain in a 64-year-old woman diagnosed with trigeminal neuralgia disappeared with the administration of carbamazepine, but carbamazepine had to be discontinued because of intolerable lassitude and liver dysfunction. Afterward, the paroxysmal pain reoccurred, and depressive symptoms appeared. Milnacipran was then administered at a dosage of 50 mg/d for 2 months, and the paroxysmal pain and depression disappeared completely. Carbamazepine is the drug of first choice for trigeminal neuralgia, but the present results suggest that milnacipran is worth investigating for patients who do not respond to carbamazepine, who cannot stay on carbamazepine because of side effects, and who exhibit depressive symptoms.

Key Words: milnacipran, trigeminal neuralgia, serotonin noradrenaline reuptake inhibitor

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Trigeminal neuralgia is a condition characterized by paroxysmal excruciating pain unique to the trigeminal system. Its pathophysiology is thought to be abnormal discharges caused by demyelination of trigeminal sensory fibers. Carbamazepine, an antiepileptic drug, is the drug of first choice in the treatment of trigeminal neuralgia. Carbamazepine is said to inhibit cell membrane excitation by acting to block sodium channels, thereby suppressing neuronal activity and inhibiting paroxysmal pain.

Milnacipran, a serotonin noradrenaline reuptake inhibitor, has recently been reported to be effective against several painful diseases. We report a patient in whom carbamazepine was effective against paroxysmal pain but had to be discontinued because of side effects of lassitude and liver dysfunction. Discontinuation of carbamazepine was followed by recurrence of the paroxysmal pain and the presentation of depressive symptoms. The patient was then given milnacipran at a dosage of 50 mg/d, with which the paroxysmal pain disappeared completely and the depressive symptoms improved.

CASE REPORT

The patient was a 64-year-old female homemaker. Her main complaint was excruciating pain in the left mandible when she washed her face. One year and 3 months before her first medical examination, she had become aware of paroxysmal pain near the left mental foramen when she washed her face. At that time, she experienced pain, which felt like a knife cut, for less than 30 seconds only when washing her face in the morning and evening. She felt as if her left under jaw cracked during the paroxysms of pain. There were no problems at other times, and the frequency of pain attacks remained almost unchanged; therefore, she did nothing for 10 months. Later, the pain gradually began appearing when she was eating or talking. It continued and worsened until it became unbearable, and she visited a dentist. She was told at 1 dental clinic that there was nothing wrong with her teeth, but, at another, she was diagnosed with pulpitis of the lower left second molar and underwent pulpectomy and root canal treatment for 2 months. However, the symptoms remained unchanged. Although loxoprofen sodium was prescribed, it did not bring her any improvement of pain. She felt as if she was going to faint away with pain and could do nothing but freezing during the paroxysms of pain. After that, she was referred to our hospitals.

From the clinical findings that matched the criteria of the International Headache Society,[1] she was diagnosed with trigeminal neuralgia (left third branch) and given carbamazepine at a dosage of 200 mg/d. This was continued for 1 week, and

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the paroxysmal pain disappeared completely. However, drowsiness and severe lassitude appeared, and she refused the carbamazepine. Moreover, biochemical examination revealed elevation of γ-guanosine triphosphate to 216, so the administration of carbamazepine had to be discontinued because of suspected drug-induced hepatic dysfunction. After discontinuation, the frequency of the paroxysmal pain increased until it was the same as before treatment.

Meanwhile, percussion pain of the left lower molar appeared, and the tooth was extracted after it was judged that it could not be preserved. She had hoped that extraction of the tooth would lead to disappearance of the paroxysmal pain, but the symptoms were unchanged. The symptoms of irritation, depressive mood, and loss of appetite appeared after the fear of pain induced by eating or other activities, and sulpiride of 150 mg/d and alprazolam of 1.2 mg/d were administered for 4 weeks. Mild improvement was seen in the depressive symptoms, but there was no improvement in the paroxysmal pain. Milnacipran, which has been reported to be effective against several painful diseases, was then begun at 15 mg/d. After 1 week, the frequency of paroxysmal pain lessened, and the dosage was gradually increased to 50 mg/d. Domperidone of 20 mg/d was coadministered for 5 months for nausea that was thought to be a side effect. Two months after the start of milnacipran, the paroxysmal pain had disappeared completely. At the same time, her depressive symptoms also disappeared. It became possible for her to wash her face, brush her teeth, and eat without difficulty. The dosage of 50 mg/d was maintained for 6 months afterward and then gradually decreased to 30 mg/d without domperidone. There was no recurrence, and the course remained good without any side effects. The existence of a neoplastic lesion was ruled out with a head magnetic resonance imaging examination.

DISCUSSION

The most common cause of trigeminal neuralgia is focal compression of the trigeminal nerve root, close to its point of entry into the pons, by an aberrant loop of artery or vein.[2] Although many patients have adequate relief of symptoms when treated with carbamazepine, some patients require surgical treatment of microvascular decompression because their symptoms are intractable or because they cannot tolerate the medications.[3]

To the authors' knowledge, there are no reports on the effectiveness of the antidepressant milnacipran for trigeminal neuralgia. Looking at antidepressants, we find only reported cases of effectiveness of amitriptyline, a tricyclic antidepressant,[4] and paroxetine, a selective serotonin reuptake inhibitor.[5]

Concurrent administration of baclofen, a central muscle relaxant and a γ-aminobutyric acid B receptor agonist, has been reported as a way to augment the analgesic effect of carbamazepine.[6] However, there are no reports on the action of milnacipran on γ-aminobutyric acid receptors.

Meanwhile, it has been reported that imipramine, which is a tricyclic antidepressant and acts both on serotonin and noradrenaline, exhibited an analgesic effect in an animal model of trigeminal neuralgia.[7] The mechanism of action of imipramine may be mainly mediated via noradrenergic projection to the trigeminal nucleus, although serotonergic projection may be also involved in its mechanism.[8] It is possible that milnacipran exhibits an analgesic effect through a similar mechanism.

Morphine shows an analgesic effect in animal models of trigeminal neuralgia.[7] Milnacipran is reported to enhance the effect of tramadol, an opioid receptor agonist.[9] These findings suggest a possibility that action via opioid receptors contributes to the analgesic effect of milnacipran.

Carbamazepine is the drug of first choice for trigeminal neuralgia. However, the use of milnacipran would be considered for patients who do not respond to carbamazepine, who cannot continue to take its medication because of side effects, and who exhibit paroxysmal pain and depressive symptoms.

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RGS4 is not a susceptibility gene for schizophrenia in Japanese: Association study in a large case-control population ☆

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Abstract

The regulator of the G-protein signaling 4 (RGS4) has been implicated in the susceptibility to schizophrenia. RGS4 interacts with ErbB3 that acts as receptors for neuregulin 1 and these proteins may play a role in the pathogenesis of schizophrenia via glutamatergic dysfunction. Recently, two meta-analysis studies provided different interpretations for the genetic association between *RGS4* and schizophrenia. We attempted to confirm this association in a case-control study of 1918 Japanese patients with schizophrenia and 1909 Japanese control subjects. Four widely studied single nucleotide polymorphisms (SNPs) were genotyped, and none showed association with schizophrenia. SNP 1 (rs10917670), $p=0.92$; SNP 4 (rs951436), $p=0.91$; SNP 7 (rs951439), $p=0.27$; and SNP 18 (rs2661319), $p=0.43$. A haplotype block constructed by these SNPs spans the 5′ flanking region to the 5′ mid-region of the *RGS4* gene. Previous meta-analysis showed that both two major haplotypes of this block were risk haplotypes. The two common haplotypes were observed in the Japanese population. However, neither haplotype was significantly associated with schizophrenia. We conclude that the common haplotypes and SNPs of the *RGS4* gene identified thus far are unlikely to contribute to the genetic susceptibility to schizophrenia in the Japanese population.
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Keywords: Schizophrenia; RGS4; Japanese; Haplotype; Case-control study

1. Introduction

The regulator of G-protein signaling 4 (RGS4) is one of 28 subtypes of the RGS family. The molecule activates guanine triphosphatase (GTPase) and regulates synaptic signaling particularly in glutamatergic neurons, and is suggested to be involved in one of the most important

☆ DNA sequences and GenBank Accession numbers. Regulator of G-protein signaling 4 (GenBank accession no. AB209019, BT007025, BC051869, AF4939).

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pathways underlying vulnerability to schizophrenia. *RGS4* mRNA is widely distributed but is dense in the cortical layers of the brain. Gene expression profiling/scan of human postmortem brain tissues has revealed a significant decrease in *RGS4* gene expression in schizophrenia-affected prefrontal cortex, and motor and visual cortices (Mirnics et al., 2001). Decreased RGS4 protein levels have been reported in other brain regions, including the cingulate gyrus, superior frontal gyrus, and insular cortex (Erdely et al., 2006). This phenomenon was not observed in monleys treated with antipsychotics (Kampman et al., 2006). Therefore, a decrease in *RGS4* expression appears to be a specific change in schizophrenia. Although *Rgs4* mutant mice do show intact prepulse inhibition (Grillet et al., 2005), this does not exclude the possible role of RGS4 in schizophrenia.

Some studies have shown linkage between schizophrenia and chromosome 1q23.3, where *RGS4* is localized (Brzustowicz et al., 2000; Brzustowicz et al., 2002; Lewis et al., 2003). Transmission disequilibrium test (TDT) showed a genetic association of four SNPs, designated as SNP1, 4, 7, and 18, and their constructed haplotypes to schizophrenia (Chowdari et al., 2002). These markers were located at the 5′ flanking region and intron 1 of *RGS4*, and in high-linkage disequilibrium, spanned approximately 6 kb. This association may explain functional differences associated with gene expression. There are two major haplotypes, GGGG and ATAA, either of which had been reported to be a risk haplotype for schizophrenia. However, which is the risk haplotype differs according to the population analyzed in the initial study and also in the following replication studies. Recently, two meta-analysis studies provided different interpretations for the genetic association between *RGS4* and schizophrenia (Li and He, 2006; Talkowski et al., 2006).

As the most straightforward way to determine a genetic association is to perform an analysis with sufficient statistical power to show the association. In this study, we attempted to confirm this association in a large Japanese population. This is the first association study of schizophrenia in the Japanese population.

2. Materials and methods

2.1. Subjects

All subjects were of Japanese descent and were recruited from the main island of Japan. A total of 1918 unrelated patients with schizophrenia (mean age $\pm$ SD: 48.9 ± 14.5 years, 1055 male and 863 female) received diagnosis according to the Diagnostic and Statistical Manual of Mental Disorders, Fourth Edition (DSM-IV). Control subjects were 1909 mentally healthy unrelated subjects (mean age $\pm$ SD: 49.0 ± 14.3 years, 1012 male and 893 female) with family histories of mental illness within second-degree relatives as self-reported. The present study was approved by the Ethics Committees of the University of Tsukuba, Niigata University, Fujita Health University, Nagoya University, Okayama University and Teikyo University, and all participants provided written informed consent.

2.2. Genotyping

DNA was extracted from blood samples. We genotyped four SNP markers, rs10917670 referred to as SNP1, rs951436 as SNP4, rs951439 as SNP7 and rs2661319 as SNP18, and constructed the risk haplotype reported in previous studies. The former three SNPs were genotyped by TaqMan assay, and the fourth was genotyped by the restriction fragment length polymorphism (RFLP) method. Predesigned TaqMan SNP genotyping assays were selected from the Applied Biosystems database (http://www.appliedbiosystems.com), C_9619634_10 for rs951436, C_344532_10 for rs951439 and C_16265754_10 for rs2661319. The TaqMan reaction was performed in a final volume of 3 μl consisting of 2.5 ng genomic DNA and Universal Master Mix (EUROGENTEC, Seraing, Belgium), and genotying was performed with an ABI PRISM 7900HT Sequence Detection System (Applied Biosystems, Foster City, CA, USA).

Genotyping of the rs10917670 was determined with the RFLP method after polymerase chain reaction (PCR) amplification of the region including the polymorphism with primers 5′-GGTGTCATG-GAAAGTGCTTG-3′ and 5′-GGCACAGAACAGGG-GAAATA-3′. PCR was carried out at 94 °C predenature for 10 min, followed by 20 cycles of 94 °C denature for 20 s, $65(-0.2 \times n$ cycle done) °C annealing for 30 s and another 20 cycles of 94 °C for 20 s, 61 °C for 30 s, and 72 °C for 30 s, followed by 72 °C final extension for 1 min. The reaction was performed in GoldBuffer with 2.5 mM $MgCl_2$, 0.16 mM dNTP and 0.5 U AmpliTaq Gold Polymerase (Applied Biosystems, Foster City, CA) in a GeneAmp PCR System 9700 (Applied Biosystems). PCR amplicons were digested with *Mva*I for 4 h, and electrophoresed on agarose gels to separate according to size. Genotypes of randomly selected subjects, as determined by PCR-RFLP, were confirmed by direct resequencing with a BigDye Terminator Cycle Sequencing FS Ready Reaction Kit (Applied Biosystems) in an ABI3100 autosequencer.

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