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).

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.

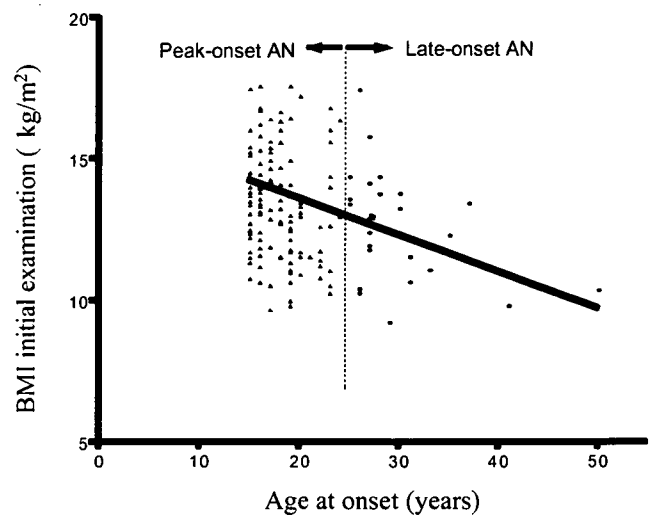

Figure 1. Relationship between age at onset and body mass index (BMI) at the time of initial examination. A significant linear correlation was seen ($y = 15.6 - 0.12x$; $r^2 = 0.064$; $P = 0.0018$). (▲) Peak-onset anorexia nervosa (AN); (●) late-onset AN.

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

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.

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±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±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′-GGTGTCATGGAAAGTGCTTG-3′ and 5′-GGCACAGAACAGGGGAAATA-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×*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 *MvaI* 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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Table 1
Distribution of the polymorphisms in the *RGS4* gene

SNP marker	Controls			Minor allele frequency		Schizophrenics			Minor allele frequency	Statistics Genotype	Statistics Allele	
										p value	X^2	*p* value
rs10917670	aa	ag	gg			aa	ag	gg				
(*n*=1815)	20.2%	49.3%	30.5%	0.455	(*n*=1805)	21.7%	48.0%	30.8%	0.449	0.594	0.30	0.584(0.924)
rs951436	gg	gt	tt			gg	gt	tt				
(*n*=1918)	21.9%	48.6%	29.5%	0.468	(*n*=1918)	21.3%	51.0%	27.6%	0.462	0.554	0.33	0.566(0.909)
rs951439	gg	ga	aa			gg	ga	aa				
(*n*=1904)	30.1%	50.8%	19.1%	0.427	(*n*=1911)	32.5%	50.0%	17.9%	0.445	0.112	2.47	0.116(0.270)
rs2661319	gg	ga	aa			gg	ga	aa				
(*n*=1911)	14.0%	45.3%	40.7%	0.381	(*n*=1915)	13.8%	48.5%	37.7%	0.367	0.212	1.63	0.202(0.429)

Genotype-based association was tested with Cochran-Armitage test for trend.
Allele-based association was tested: nominal *p* values (and corrected *p* values for multiple testing (with permutation test)) were shown.

2.3. Statistical analysis

The Hardy–Weinberg equilibrium, linkage disequilibrium and allelic/haplotype frequencies, as well as an association between SNP or haplotype and schizophrenia, were determined with the Haploview software program (http://www.broad.mit.edu/mpg/haploview/). Permutation tests were also performed to calculate corrected *p* values for multiple testing by the Haploview software. Genotype-based association was tested with Cochran–Armitage test for trend. Statistical significance was accepted at $p<0.05$.

3. Results

Genotypic distributions of the four SNPs among the subject groups are shown in Table 1. Distributions of all four SNPs did not differ from the Hardy–Weinberg equilibrium. No association with schizophrenia was detected with the rs10917670 ($p=0.58(0.92)$), rs951436 ($p=0.57(0.91)$), rs951439 ($p=0.12(0.27)$), and rs2661319 ($p=0.20(0.43)$). These four SNPs were in linkage disequilibrium each other ($D'=0.84$–0.97, $r^2=0.35$–0.72) (Table 2). ATAA and GGGG were the major haplotypes of the gene, as observed in other ethnic groups. The frequencies of these haplotypes did not differ significantly between the schizophrenia and control groups (Table 3).

Table 2
Linkage disequilibrium between SNPs in Japanese population

	rs10917670	rs951436	rs951439	rs2661319
rs10917670		0.85	0.87	0.84
rs951436	0.52		0.96	0.97
rs951439	0.72	0.62		0.96
rs2661319	0.35	0.65	0.42	

Values at upper right show D' and those at lower left do R^2.

4. Discussion

Talkowski et al. (2006) reported a meta-analysis of *RGS4* polymorphisms in schizophrenia based on genotype data from 13,807 individuals. Although significant associations with individual SNPs/haplotypes were not observed, global analysis revealed significant transmission distortion, in particular, overtransmission of two common haplotypes that account for the vast majority of all haplotypes. In their meta-analysis of case-control associations, a modest association was detected when the two common haplotypes were combined and compared against all other haplotypes combined (frequency of two common haplotypes/frequency of rare haplotypes: cases, 0.834/0.166; control samples, 0.817/0.183; $p=0.09$). This association was markedly significant in a Chinese population (cases, 0.857/0.143; control samples, 0.518/0.482; $p=0.0001$) (Talkowski et al., 2006), although another Chinese case-control study did not show this association (Guo et al., 2006). In the present study, the frequencies of these haplotypes were similar between the two groups (cases, 0.742/0.258; control samples, 0.747/0.253).

Talkowski et al. (2006) reported that separate analyses of 3486 cases and 3755 controls samples detected a

Table 3
Haplotype distribution of the RGS4 gene

Haplotype	Frequeicy in the controls	Frequency in the schizophrenics	X^2	*p* value
ATAA	0.396	0.410	1.39	0.239(0.782)
GGGG	0.346	0.337	0.62	0.431(0.970)
GTGA	0.089	0.090	0.01	0.911(1.00)
GGGA	0.083	0.089	1.18	0.277(0.847)
GTAA	0.025	0.024	0.20	0.654(1.000)
AGGG	0.027	0.019	5.83	0.016(0.066)
ATGA	0.016	0.011	3.22	0.073(0.313)

Nominal *p* values and corrected *p* values for multiple testing (in parenthesis) were shown.

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significant association with rs951436 (p=0.01). In a Chinese population, significant association of the G allele of rs951436 was reported (OR=1.54, p=0.002) (Talkowski et al., 2006), although another study did not report this association (OR=1.19, p=0.11) (Guo et al., 2006). We calculated the power of our study on the basis of the genotype data. With a genetic relative risk of 1.15, and assuming an α value of 0.05 and a risk allele frequency of 0.4, the sample size of the present study had a power of 0.92. Therefore, the present study could validly conclude that an association between SNP 4 and schizophrenia is not likely, even in East Asian populations.

The *RGS4* gene is small, spanning approximately 7 kb, and the four SNPs that have been reported to be associated with schizophrenia are included in one haplotype block with two major haplotypes in the Caucasian and East Asian populations (http://www.hapmap.org/cgi-perl/gbrowse/hapmap_B35/). The haplotype block extends from the 5′ flanking region to the 5′ midregion of the *RGS4* gene. Subjects of the meta-analysis of Talkowski et al. (2006) numbered 13,807, and subjects in our study numbered 3821. Neither study indicates a contribution of the haplotype block to the genetic susceptibility to schizophrenia. Results of the present study clearly exclude the 5′ genomic region of the *RGS4* gene in the susceptibility to schizophrenia. However, this does not exclude the possibility of genetic variation(s) in the 3′ region of the gene. Further analysis, particularly resequencing of this region, is necessary.

5. Author disclosure

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Author Ishiguro designed the study, ran the experiment and wrote the manuscript. Author Horiuchi and Koga prepared the sample analyzed. Author Inada, Iwata, Ozaki, Ujike, Muratake, Someya, managed the sample collection. Author Arinami undertook the statistical analysis and supervised this study.

No author has conflict of interest.

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No association between the glutamate decarboxylase 67 gene (GAD1) and schizophrenia in the Japanese population

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Abstract

Postmortem studies regarding schizophrenia revealed altered expression of genes related to γ-amino butyric acid neurotransmission system. One of the most consistent findings is the reduced level of 67 kDa glutamic acid decarboxylase isoform (GAD_{67}). Moreover, several studies reported positive associations between the GAD_{67} gene (GAD1) and schizophrenia. These reasons, motivated us to carry out replication study regarding association between GAD1 (fourteen tagging SNPs) and schizophrenia in Japanese population (562 schizophrenic patients and 470 controls). However we couldn't confirm significant association that had been previously reported. Considering size of our sample and strategy that corresponds well with the approaches used in gene-based association analysis, our conclusion is that GAD1 does not play a major role in schizophrenia in Japanese population.

Keywords: Schizophrenia; GAD1; Linkage disequilibrium; GABA; Association study

1. Introduction

Abnormalities in the γ-amino butyric acid (GABA) neurotransmission system are thought to be involved in the pathophysiology of schizophrenia. Several postmortem studies showed altered expression of genes related to GABA in schizophrenic patients. One of the most consistent findings is related to reduced level of 67 kDa glutamic acid decarboxylase isoform (GAD_{67}), key enzyme in GABA synthesis (Lewis et al., 2005).

The GAD_{67} is encoded by the glutamic acid decarboxylase 1 gene (GAD1) located on 2q31, and two studies reported positive associations between GAD1 and schizophrenia (Straub et al., 2003; Addington et al., 2005). Interestingly, those studies showed positive association of: 1) childhood onset schizophrenia (COS) and 2) cortical gray matter volume loss with schizophrenia (Addington et al., 2005). Moreover, a

Abbreviations: γ-amino butyric acid, (GABA); 67 kDa isoform of glutamic acid decarboxylase, (GAD_{67}); Glutamic acid decarboxylase 1 gene, (GAD1); Childhood onset schizophrenia, (COS); Adult onset schizophrenia, (AOS); Single nucleotide polymorphism, (SNP); Linkage disequilibrium, (LD); Minor allele frequency, (MAF); Genotype relative risk, (GRR); Age at onset, (AAO).

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significant association between GAD1 and schizophrenia in two independent adult onset schizophrenia (AOS) samples has been reported as well, suggesting biological continuity between COS and AOS (Straub et al., 2003).

In contrast, three other studies reported no association between GAD1 and schizophrenia, although, due to sample size, possibility of type II error cannot be excluded (De Luca et al., 2004; Lundorf et al., 2005; Zhang et al., 2005).

It is widely accepted that there are limitations in interpreting the results of simple replication studies that examine the same or a smaller number of single nucleotide polymorphisms (SNPs) as in the original study. Aforementioned can be explained by the differences in allele frequency or variation of linkage disequilibrium (LD) structure (population dependence). To overcome these limitations, a gene-based approach rather than an SNP-based or haplotype-based approach is currently recommended (Neale and Sham, 2004). In such studies it is important to: (1) include both gene and gene flanking regions in testing for association, and (2) select genetic variants which adequately reflect the LD background in the targeted population (e.g. tagging SNPs).

Applying this gene-based association concept, we tested the association between GAD1 tagging SNPs and schizophrenia using relatively large samples in the Japanese population.

2. Materials and methods

2.1. Subjects

The sample used in this study was comprised of 562 schizophrenia patients (301 males and 261 females; range=15–82 years old, median=44 years old, mean± SD=44.9±15.2 years) and 470 healthy controls (269 males and 201 females; range=19–95 years old, median=34 years old, mean±SD=37.5±14.6 years). All subjects were unrelated and with Japanese ethnicity. Subject attributes and psychiatric assessment were identical to those described elsewhere (Ikeda et al., 2005).

Subsequent to study description, written informed consent was asked from each subject. This study was approved by the Ethics Committee at Fujita Health University and Nagoya University.

2.2. SNP selection

We first included marginal and significant SNPs (Markers 4, 5, and 6) reported by others (Addington et al., 2005) and a potent functional SNP (Marker 3; located 2246 base pairs (bp) upstream from the initial exon and Marker 12; located in 3′UTR) (Table 1). Next, we consulted the HapMap database (release#21, population: Japanese in Tokyo (JPT), minor allele frequency (MAF): more than 0.01). In this step, we determined the boundaries of the GAD1 gene that covered 5′-flanking regions including 5000 bp from the initial exon and 1700 bp downstream (3′) from the last exon (GenBank accession No. NM_000817: Supplementary Figure 1). Then eleven 'tagging SNPs' were selected with the criterion of an r^2 threshold greater than 0.8 in 'pair-wise tagging only' mode using the 'Tagger' program (de Bakker et al., 2005), implement of HAPLOVIEW software (Barrett et al., 2005). These tagging SNPs were used in the subsequent association analysis (since Markers 4 and 6 were listed in the HapMap data, they were force-included with these 'tagging SNPs'). Overall, 14 tagging SNPs were examined.

2.3. SNP genotyping

All SNPs were genotyped by TaqMan assay (Applied Biosystems Japan Ltd, Tokyo). Detailed information, including reaction conditions, can be seen in another paper (Ikeda et al., 2005).

2.4. Statistical analysis

Marker-trait association was evaluated by the χ^2 test (allele and genotype-wise analyses). For haplotype-wise analysis, LD blocks were initially defined in accordance with Gabriel's criteria, and haplotype frequencies were estimated in each LD block with the Expectation–Maximization algorithm. Log likelihood ratio tests were performed for global *P*-values (COCAPHASE 2.403 program, Dudbridge, 2003). Power calculation was performed with a web-based statistical program, Genetic Power Calculator (Purcell et al., 2003). Power was estimated under multiplicative model of inheritance, assuming the disease prevalence to be 1% and the population susceptibility allele frequencies to be the values in observed in control samples.

3. Results

All genotype frequencies of these SNPs were in Hardy–Weinberg equilibrium. The LD matrices of the 14 tagging SNPs we tested are shown in Supplementary Figure 2. No association was found between cases and controls in allele-, genotype- or haplotype-wise analyses (Table 1).

Table 1
Association analysis of tagging SNPs in GAD1

SNP ID[a]		Positions[b]	Blocks[c]	Genotype distribution[d]								MAFs[e]			*P*-value		
				N		M/M		M/m		m/m							
				SCZ	CON	SCZ	CON	SCZ	CON	SCZ	CON	SCZ	CON		Genotype	Allele	Haplotype
Marker 1	rs1978340	1	I	561	466	371	306	175	148	15	12	.18	.18	(.31)	.978	.915	.549
Marker 2	rs3762561	625	I	561	469	368	292	165	158	28	19	.20	.21	(0)	.297	.500	
Marker 3	rs12185692	704		561	466	269	237	244	194	48	35	.30	.28	(.37)	.610	.328	
Marker 4	rs3749034*	3353	II	561	466	244	211	265	216	52	39	.33	.32	(.16)	.795	.517	.194
Marker 5	rs2270335*	4574	II	561	466	245	212	265	214	51	40	.33	.32	(0)	.836	.574	
Marker 6	rs2241165*	8257	II	561	466	244	212	265	215	52	39	.33	.31	(.19)	.768	.484	
Marker 7	rs3828275	12618		561	467	266	224	247	200	48	43	.31	.31	(.49)	.894	.980	
Marker 8	rs2241164	17437		562	470	189	142	271	243	102	85	.43	.44	(.31)	.459	.444	
Marker 9	rs769407	23586	III	561	468	314	261	215	179	32	28	.25	.25	(.24)	.982	.900	.438
Marker10	rs3791851	28552	III	561	467	298	238	226	191	37	38	.27	.29	(.25)	.582	.350	
Marker 11	rs3791850	37978	III	561	468	431	370	119	93	11	5	.13	.11	(.18)	.429	.275	
Marker 12	rs769395	46681	III	561	466	287	233	237	193	37	40	.28	.29	(.27)	.484	.431	
Marker 13	rs16858996	48980		561	468	370	295	176	153	15	20	.18	.21	(.11)	.302	.197	
Marker 14	rs17701824	49124		561	468	266	221	231	204	64	43	.32	.31	(.43)	.458	.622	

Numbers in parenthesis represent MAFs in Caucasians (cited from HapMap database and Applied Biosystems database).

[a] IDs with asterisk represent significant or marginally significant SNPs in Addington's report.

[b] Based on Accession No. NT005403.16.

[c] Determined by HAPLOVIEW.

[d] *N* = number, M = major allele, m = minor allele, SCZ = schizophrenia, CON = control.

[e] MAFs = minor allele frequencies.

Power analyses showed that the power was more than 80% when genotype relative risk (GRR) was set at 1.3–1.5 under a multiplicative model of inheritance.

4. Discussion

In this study, we were unable to confirm an association between GAD1 and schizophrenia in the Japanese population.

Addington et al. (2005), using an *in silico* approach, reported that positive SNPs in the 5′ region (Marker 4 and 5 in our study) of GAD1 possibly have various effects on gene expression. They speculated that these SNPs may alter the expression level of GAD1. Their findings supported previous postmortem studies showing down-regulation of GAD67 in schizophrenics. This data suggests that the 5′-flanking region of this gene might harbor schizophrenia-susceptibility factor. However, our results do not support the positive association (Markers 4 and 5) in the 5′-flanking region of GAD1 previously reported by Addington, even though we examined the other three SNPs in the 5′-flanking region (Markers 1, 2 and 3) in addition to Addington's two SNPs. Our sample size also showed quite high power. From the viewpoint of the common disease-common variant hypothesis (Chakravarti, 1999), our data didn't provide the evidence that GAD1 have a major role in schizophrenia in the Japanese population.

We also included an explorative analysis of gender effect and age at onset (AAO: $N=310$), for the following reasons: 1) Straub et al. (2003) and Addington et al. (2005) reported that relations were significantly greater or stronger in females, and 2) this analysis allowed us to consider the relation between the negative results of our AOS samples in Japanese and the positive COS samples in Caucasians. However, no associations were found in analysis subdivided by gender, or between AAO and GAD1 allele and haplotypes (evaluated by haplotype trend regression analysis; Liu and Muse, 2005: data not shown).

Although the strategy we used for the present association analysis corresponded well with that used in gene-based association analysis (Neale and Sham, 2004), several limitations must be outlined. First, in case of examination of possible association between GAD1 and schizophrenia, causal variants with extremely rare MAFs, allelic heterogeneity, or locus heterogeneity should be considered (Neale and Sham, 2004). In such situations, quite large sample sizes are needed for rare variants searching; i.e. more than 3500 cases and controls are required for 80% power when GRR is set at 1.5 and MAF is 1% (Purcell et al., 2003). Second, our control subjects were significantly younger than case subjects. Thus, our control samples may have included a number of individuals below the age of peak risk for schizophrenia-onset, and this confounding factor has potential for decreasing power of this study. Third, GAD1 may influence the function and morphology of the dorsolateral prefrontal cortex (Addington et al., 2005; Lewis et al., 2005). Therefore, endophenotypic approaches such as cognitive function, brain imaging and other phenotypes reflecting characteristics of GAD1 will be needed in the future (Gottesman and Gould, 2003).

In conclusion, our gene-based analysis of GAD1 showed no association between this gene and AOS in the Japanese population. Further studies using different population samples will be needed to conclude whether GAD1 is a race specific or rare phenotype specific susceptibility factor for AOS.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.schres.2006.12.020.

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Possible association of *β-arrestin 2* gene with methamphetamine use disorder, but not schizophrenia

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Recent investigations suggest that the AKT/glycogen synthase kinase 3 (GSK3) signaling cascade may be associated with the pathophysiology of schizophrenia and methamphetamine (METH) use disorder. One important molecule related to this cascade is β-arrestin 2 (ARRB2). We therefore conducted a genetic case–control association analysis of the gene for *ARRB2* with schizophrenia and METH use disorder in a Japanese population (547 people with schizophrenia, 177 with METH use disorder and 546 controls). A possible association of 'tag single nucleotide polymorphisms (SNPs)' was found in METH use disorder (rs1045280: $P_{genotype} = 0.0118$, $P_{allele} = 0.00351$; rs2036657: $P_{allele} = 0.0431$; rs4790694: $P_{genotype} = 0.0167$, $P_{allele} = 0.0202$), but no association was found with schizophrenia. We also evaluated the gene–gene interactions among ARRB2, AKT1, and GSK3B, which we previously reported for each of these diseases. However, no interaction was seen in our samples. This is the first association analysis of ARRB2, and our results indicate that ARRB2 may play a role in the pathophysiology of METH use disorder.

Keywords: AKT, GSK3, methamphetamine use disorder, schizophrenia, β-arrestin

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Dopamine D2 receptors are the main target of therapeutic agents for psychiatric diseases. So far, D2 receptors have been thought to inhibit cyclic AMP (cAMP) synthesis through interaction with Gαi/o and negatively regulate the activity of protein kinase A. However, several investigations showed that new intracellular proteins can facilitate some aspects of D2 receptor signaling, such as the AKT/glycogen synthase kinase 3 (GSK3) signaling cascade (Bonci & Hopf 2005). This AKT1–GSK3 signaling system has been discussed as a major target for lithium action, and it has been hypothesized that this system is involved in the pathophysiology of mood disorders (Gould & Manji 2005).

Recently, two *in vivo* studies using dopamine transporter knock-out (KO) mice or amphetamine administration showed that the AKT/GSK3 cascade partially mediates dopamine-associated behaviors (Beaulieu *et al.* 2004; Emamian *et al.* 2004). In terms of the relation between AKT1/GSK3 and schizophrenia, the convergent evidence from animal, postmortem and genetic studies has been reported (Emamian *et al.* 2004). Two genetic replication studies revealed significant associations of AKT1 with schizophrenia (Ikeda *et al.* 2004; Schwab *et al.* 2005), although our previous study of the GSK3β (*GSK3B*) gene did not support such an association in a Japanese population (Ikeda *et al.* 2005b).

Elsewhere, classical studies in Japan (Tatetsu *et al.* 1956) and UK (Connell 1958) showed that methamphetamine (METH) consumption induces psychosis at a high rate (92 and 100%, respectively). Because the symptomatology of METH-induced psychosis is similar to that of schizophrenia (especially paranoid type), METH-related disorders may also involve dopaminergic abnormalities. In support of this speculation, we reported an association between single nucleotide polymorphism (SNP) and haplotypes in AKT1 and METH use disorder (Ikeda *et al.* 2005c). AKT1 and GSK3β would therefore seem to be prime candidate genes for schizophrenia and METH use disorder, while the related molecules (or genes) of this cascade are also considered to be candidate factors for these disorders.

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A recent study showed that β-arrestin 2 (ARRB2) is an important mediator of the AKT/GSK3 cascade introducing dopamine-associated behaviors: ARRB2 KO mice exhibit significantly less pronounced locomotor activation than wild-type mice following the administration of amphetamine, and double mutation ARRB2/dopamine transporter KO mice display significantly lower locomotor activity than dopamine transporter KO mice (Beaulieu *et al.* 2005). This study also showed that ARRB2 interacts with AKT and protein phosphatase 2 A (PP2A) and that these signaling complexes are regulated by dopamine. According to this evidence, ARRB2 seems to be a promising candidate as a molecule related to the AKT/GSK3 signaling cascade and/or the pathophysiology of schizophrenia and METH use disorder.

We conducted this association analysis of the *ARRB2* gene (located on 17p13) with schizophrenia and METH use disorder in a Japanese population. We first evaluated the linkage disequilibrium (LD) structure of this gene and selected 'tag SNPs'. These 'tag SNPs' were then used to reflect the LD properties in the Japanese population in the following association analysis.

Materials and methods

Subjects

Five hundred and forty-seven patients with schizophrenia [283 males and 264 females: mean age ± standard deviation (SD) 45.6 ± 16.0 years] and 177 patients with METH use disorder (all patients were diagnosed as having METH dependence, 145 males and 32 females: mean age ± SD 36.8 ± 12.0 years) participated in this study. A total of 546 healthy controls (255 males and 291 females: mean age ± SD 37.0 ± 14.4 years) were recruited as control subjects. The subjects for the 'LD evaluation' were 96 controls who were also subjects in the association analysis. All subjects were unrelated each other and ethnically Japanese.

The patients were diagnosed according to DSM-IV or ICD-10DCR criteria with the consensus of at least two experienced psychiatrists on the basis of unstructured interviews and review of medical records. All healthy controls were also psychiatrically screened based on unstructured interviews.

Among the subjects with METH use disorder, 164 have a comorbid diagnosis of METH-induced psychosis, three of anorexia nervosa, one of obsessive-compulsive disorder and one of major depressive disorder. In addition, 129 subjects with METH use disorder or abuse have a dependence on drugs other than METH. Subjects with METH use disorder were excluded if they had a comorbid diagnosis of any psychotic disorder other than METH-induced psychosis. More detailed characterizations of these subjects were published elsewhere (Ikeda *et al.* 2005a; Nishiyama *et al.* 2005).

After description of the study, written informed consent was obtained from each subject. This study was approved by the Ethics Committee at Fujita Health University School of Medicine, Nagoya University Graduate School of Medicine and each participating institute of the Japanese Genetics Initiative for Drug Abuse (JGIDA).

SNP selection and LD evaluation of ARRB2

We first consulted the HapMap database (release#16c.1, June 2005, http://www.hapmap.org) and SNPBROWSER2.0 database (Applied Biosystems, Foster City, CA, USA) and selected five SNPs (SNP1: rs4790689, SNP2: rs1973555, SNP3: rs1045280, SNP4: rs2036657 and SNP5: rs4790694) with minor allele frequencies (MAFs) of more than 0.05 for LD evaluation (Supplemental Table S1).

Next, we genotyped these five SNPs using our own control samples to confirm the LD structure. In this step, we selected 'tag SNPs' with criteria on r^2 threshold greater than 0.8 in pairwise tagging mode using the TAGGER program (Paul de Bakker, http://www/broad.mit.edu/mpg/tagger/), an implement of the HAPLOVIEW software program (Barrett *et al.* 2005) for the following association analysis.

SNP genotyping of ARRB2

All SNPs were genotyped by TaqMan assay (Applied Biosystems). Further detailed information, including reaction conditions, can be seen in another paper (Ikeda *et al.* 2005a).

Statistical analysis

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

Marker-trait association analysis was used to evaluate allelic and genotypic associations with χ^2 test or Fisher's exact test (SPSS 10.0 J, SPSS Japan Inc, Tokyo, Japan), and haplotypic association was investigated with log-likelihood ratio test (COCAPHASE 2.403). A more detailed description is given in our previous paper (Ikeda *et al.* 2005a).

We estimated the power of association for our sample size using GENETIC POWER CALCULATOR software (Purcell *et al.* 2003) with an α of 0.05 and a disease prevalence of 0.01.

To avoid false negative results, we did not correct for multiple testing, because Bonferroni correction is too conservative to apply to genetic association analysis (Nyholt 2001).

The significant level for all statistical tests was 0.05.

Results

For LD evaluation, five SNPs were genotyped for 96 controls. Three SNPs (SNP3–5) were selected as 'tag SNPs' by the TAGGER software (Supplemental Table S1). These genotypings of the three 'tag SNPs' were expanded for the following association analysis, and no genotype distributions in cases and controls showed deviation from HWE.