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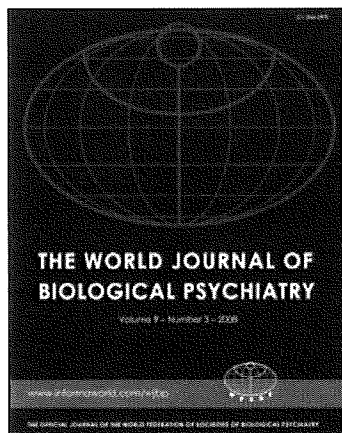
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### Abnormal microstructures of the basal ganglia in schizophrenia revealed by diffusion tensor imaging

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## BRIEF REPORT

# Abnormal microstructures of the basal ganglia in schizophrenia revealed by diffusion tensor imaging

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### Abstract

There has been a hypothesis that deficits in the basal ganglia–thalamic system may play an important role in the dysfunctional goal-directed behaviour in schizophrenia. By using diffusion tensor imaging, we measured fractional anisotropy (FA) values in the basal ganglia–thalamic system in 42 schizophrenics and 42 matched controls to investigate microstructural tissue alterations in the basal ganglia–thalamic system in schizophrenia. Schizophrenics had significantly lower FA values in the bilateral globus pallidus and left thalamus compared to controls, suggesting that schizophrenics might have microstructural abnormalities in globus pallidus and thalamus. These data support the notion that myelination abnormalities in basal ganglia–thalamic system are related to the pathophysiology of schizophrenia.

**Key words:** Schizophrenia, diffusion tensor imaging, basal ganglia, globus pallidus, MRI

### Introduction

Schizophrenia often demonstrated movement abnormalities, such as catatonia, pacing and other stereotyped behaviours considered to be associated with basal ganglia dysfunction. The basal ganglia regulates not only motor behaviours but also aspects of cognitive and limbic behaviours. There has been a hypothesis that deficits in the basal ganglia–thalamic system may play an important role in the dysfunctional goal-directed behaviour in schizophrenia (Andreasen 1999). In fact, several studies demonstrated abnormalities in the basal ganglia in schizophrenic brains, including the volume reductions of the pallidum internum of postmortem brains of patients with schizophrenia (Bogerts et al. 1985), higher volumes in the globus pallidus of previously

treated patients with schizophrenia than the healthy comparison subjects and the neuroleptic-naïve patients (Gur et al. 1998), fMRI evidence for basal ganglia dysfunction in subjects with schizophrenia (Menon et al. 2001), abnormality of oligodendroglial cells in caudate nucleus in schizophrenia (Uranova et al. 2001), and positive correlation between globus pallidus and the severity of global symptoms in neuroleptic-naïve patients (Spinks et al. 2005).

Diffusion tensor imaging (DTI) is a relatively new technique, and it is useful for evaluating white matter abnormalities in schizophrenia. We have reported progressive changes of white matter integrity in schizophrenia using DTI (Mori et al. 2007). Recently, this technique was applied to investigate

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abnormalities of the subcortical regions in neurodegenerative diseases. Patients with Parkinson's disease had significantly decreased fractional anisotropy (FA) in the region of interest along a line between the substantia nigra and the lower part of the putamen/caudate complex, in which the nigrostriatal dopaminergic neurons are lost in Parkinson's disease, demonstrating its possibility to detect microstructural tissue alterations (Yoshikawa et al. 2004). To investigate possible microstructural abnormalities in the basal ganglia–thalamic system in schizophrenia, we measured FA values in the basal ganglia and the thalami in schizophrenics and in normal controls for comparison, as a sub-analysis of our previous study (Mori et al. 2007).

## Material and methods

### *Subjects and clinical assessments*

Forty-two patients with DSM-IV schizophrenia (26 male and 16 female, one left hander, mean age:  $40.0 \pm 9.3$  years old, education:  $13.0 \pm 2.9$  years, mean duration of illness;  $16.8 \pm 9.0$  years, mean daily dose of antipsychotics (chlorpromazine equivalent):  $1005.1 \pm 735.3$  mg/day) (Association 1994) and 42 controls (26 male and 16 female, one left hander, mean age:  $39.2 \pm 9.0$  years old, education:  $17.1 \pm 3.5$  years) were participated in our study. Written informed consent was obtained from all the subjects. This study has been approved by the local ethics committee and has therefore been performed in accordance with the ethical standards laid down in the 1964 Declaration of Helsinki. All the normal subjects were screened by a questionnaire on medical history and excluded if they had neurological, psychiatric or medical conditions that could potentially affect the central nervous system. We employed the Japanese version of National Adult Reading Test (JART) as a convenient tool to measure IQ for participants (premorbid IQ for schizophrenics). Patients had fewer years of education (two-sample *t*-test,  $P < 0.0001$ ), lower scores of JART (controls:  $78.8 \pm 11.5$ , schizophrenics:  $58.7 \pm 25.3$ , two-sample *t*-test  $P < 0.001$ ).

### *Neuroimaging analysis*

MR studies were performed on a 1.5-Tesla Siemens Magnetom Vision Plus system. Axial DTI scans aligned to the plane containing anterior and posterior commissures were acquired with a pulsed-gradient, spin-echo, single-shot echo planar imaging (EPI) sequence (TR/TE = 4000/100 ms,  $256 \times 256$  matrix, FOV 240 mm,  $b = 1000$  s/mm<sup>2</sup>, NEX = 4, 20 slices, 5 mm slice thickness, 1.5 mm gap). Diffusion was measured along six non-collinear directions,

because six directions were maximum number of this Vision Plus system. For each of six gradient directions, four acquisitions were averaged. Four acquisitions without diffusion weighting ( $b = 0$ ) were also averaged. Additionally, a three-dimensional volumetric acquisition of a T1-weighted gradient echo sequence with a gapless series of thin sagittal sections using an MPRage sequence (TR/TE = 11.4/4.4 ms; flip angle, 15°; acquisition matrix,  $256 \times 256$ ; NEX = 1, FOV 315 mm; slice thickness 1.23 mm) was acquired for evaluating the volume of grey matter (GM), WM and cerebrospinal fluid (CSF) space. Seven diffusion images acquired as above by an in-house script described previously (Mori et al. 2007) on Matlab 6.5 software (Mathworks, Inc., MA, USA). Then, the FA images were spatially normalized using high-dimensional-warping algorithm (Ashburner et al. 1999) and were matched to the FA template image (Figure 1, top). To make the FA template image, we warped FA images of four normal subjects (other than 42 control subjects) to the single-subject T1 template (skull stripped image) using spatial normalization function of SPM2 and averaged the four warped FA images. The transformed FA images were smoothed with a Gaussian kernel (the filter size, full-width half-maximum:  $6 \times 6 \times 6$  mm).

Since our interest was FA changes in the basal ganglia and thalamus, we excluded other brain areas by using an explicit mask (Figure 1, top). The resultant FA maps were analyzed using Statistical parametric mapping 2 (SPM2), which implements a 'general linear model'. To test hypotheses about regional population effects, data were analyzed by a two-sample *t*-test without global normalization. JART scores were treated as nuisance variables. Furthermore, we performed correlational analyses between duration of illness, age of onset, total daily dose of antipsychotic drugs (chlorpromazine equivalent) and FA value in the schizophrenics. Our a priori hypothesis is limited to the basal ganglia; however, investigation of the FA changes within this ROI is null hypothesis. Thus, we used  $P < 0.05$ , corrected for multiple comparisons with Family-Wise Error rate (FWE) within basal ganglia as a statistical threshold.

## Results

In comparison with controls, schizophrenics had significantly lower FA values in the bilateral globus pallidus (GP) (Figure 1, bottom). Increased FA values in schizophrenics were not found in any regions (data not shown).

A correlational analysis in the schizophrenics demonstrated a significantly negative correlation

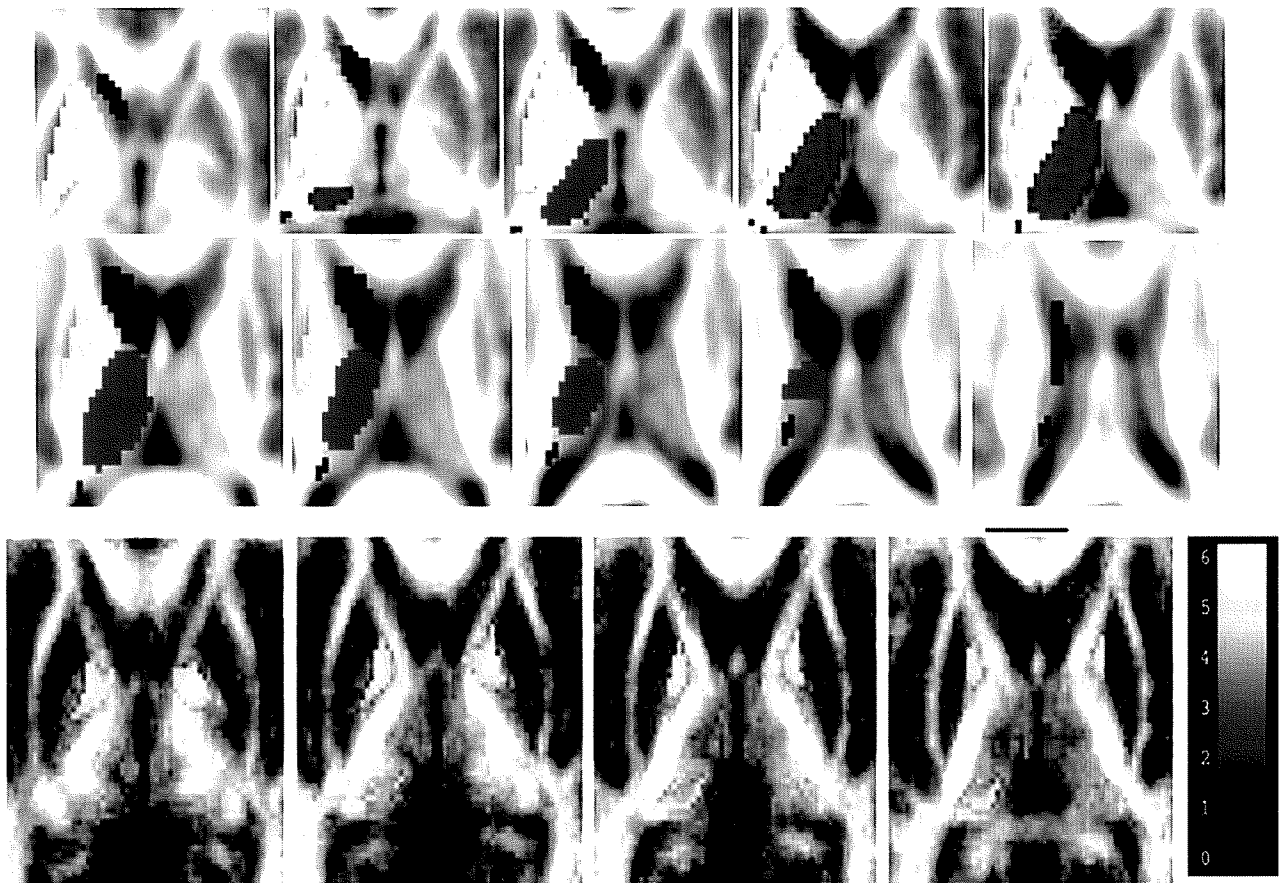


Figure 1. Top: A half of the explicit mask is displayed onto mean FA images of warped FA images obtained from 42 controls (dark blue: caudate nucleus; yellow: putamen; light blue: globus pallidus; red: thalamus). Even after averaging, the mean images are not blurred. Since globus pallidus is traversed by numerous myelinated nerve fibres, it shows higher FA value than other parts of basal ganglia. Bottom: The SPM  $\{t\}$  is displayed onto mean axial FA images of 42 schizophrenics. A significant reduction of FA value in schizophrenia was noted in the bilateral globus pallidus (right GP:  $t$  value = 6.52, Talairach coordinate  $x, y, z$ : 18, -2, -2, left GP:  $t$  value = 6.37, Talairach coordinate  $x, y, z$ : -18, -3, -2) and left thalamus ( $t$  value = 4.96, Talairach coordinate  $x, y, z$ : -18, -33, 10).

between duration of illness and FA in the left head of the caudate nucleus ( $t$  value = 4.77, Talairach coordinate  $x, y, z$ : -11, -17, -6). However, there is no significant correlation between duration of illness and FA values in the GP and the thalamus. There was no significant correlation between FA values in the basal ganglia–thalamic system with age of onset or total daily dose of antipsychotic drugs.

### Discussion

In this study, we found a significantly reduced FA value in the bilateral GP and left thalamus in schizophrenics compared to controls. We consider that reduced FA may reflect microstructural abnormalities in the basal ganglia–thalamic system in schizophrenia. A previous fMRI study suggested that GP itself may be the primary locus of the functional deficits in the basal ganglia and may be dysfunctional in schizophrenia (Menon et al. 2001). A postmortem study of basal ganglia morphology reported that only

the GP were smaller in schizophrenics than in controls (Bogerts et al. 1985). These studies indicated functional and structural abnormalities in GP in schizophrenia. Our data, reduced FA in GP in schizophrenia, were obtained using a size-adjusted high-dimensional warping method (Ohnishi et al. 2006). Our results, microstructural abnormalities in the GP in schizophrenia, are consistent with previous reports.

Although the underlying mechanisms remain to be clarified, previous DTI studies in parkinsonism have well demonstrated ongoing pathological changes in neurodegenerative diseases, suggesting that this technique has the potential to detect microstructural alterations in the basal ganglia (Yoshikawa et al. 2004). Since pathological findings of schizophrenia are still ambiguous, the underlying pathological changes of reduced FA values in schizophrenia are unclear. However, multiple lines of evidence now converge to implicate oligodendroglia and myelin in schizophrenia (Davis et al.

2003). We assume that damage of myelinated nerve fibres may contribute to FA reduction in the basal ganglia–thalamic system. The GP is traversed by numerous myelinated nerve fibres that give it the pale appearance for which it is named, and has rich connections to the putamen and the thalamus. These histological characteristics of the GP may contribute to its higher FA values. A qualitative electron microscopic study reported the density of concentric lamellar bodies (an indicator of damage of myelinated fibres) was dramatically increased in the caudate nucleus in schizophrenia, as compared to controls (Uranova et al. 2001). Such pathological changes seem to explain decreased FA values in the schizophrenic brain. However, there have been no data on whether GP also have alterations of myelinated fibres. Further pathological studies need to be conducted to draw a firm conclusion on this matter.

Although some studies demonstrated abnormalities of GP in neuroleptic-naïve schizophrenics (Spinks et al. 2005), abnormalities in the basal ganglia have been considered to relate to antipsychotic medication (Gur et al. 1998). In this study, FA changes in the GP and thalamus were not associated with the duration of illness or the daily dose of antipsychotic drugs, suggesting that FA changes in these regions might be independent of medication with neuroleptics. Guidelines for the biological treatment of schizophrenia developed by an international Task Force of the World Federation of Societies of Biological Psychiatry recommended atypical antipsychotics as first line drugs (Falkai et al. 2005, 2006). The differential treatment effects on brain morphology could be due to typical antipsychotics-associated toxicity or greater therapeutic effects of atypical antipsychotics (Lieberman et al. 2005). It would be interesting to compare patients treated with atypical antipsychotics to those with a history of typical antipsychotics treatment; however, the subgroup of patients that were only treated with atypical antipsychotics or the subgroup of patients that were only treated with typical antipsychotics were too small to investigate a possible difference between two groups in FA in our sample. To conclude whether observed change of FA value is a result of medication or relates to the pathophysiology of schizophrenia itself, longitudinal studies on treated schizophrenics, and studies on neuroleptic-naïve schizophrenics should be conducted.

There is a limitation to our study: we used a 1.5-Tesla Siemens Magnetom Vision Plus system, which is a relatively old system. We chose six gradient directions, which is quite low, as this number is the maximum number of directions in this system. Slice thickness of 5 mm and 1.5-mm slice gaps are

methodological drawbacks to this study. The reason why we used a slice thickness of 5 mm and 1.5-mm slice gaps is to cover the whole brain as in our previous study (Mori et al. 2007). There may be a partial volume effect in our mapping parameters, although we minimized the problem by using the high-dimensional warping algorithm.

Our data suggest that patients with schizophrenia might have microstructural abnormalities in globus pallidus and thalamus. The DTI study may be a promising method to investigate microstructural abnormalities in schizophrenia.

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### Declaration of Interest

None.

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## TATA Box-Binding Protein gene is associated with risk for schizophrenia, age at onset and prefrontal function

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Schizophrenia is a common polygenic disease in distinct populations, while spinocerebellar ataxia type 17 (SCA17) is a rare autosomal dominant neurodegenerative disorder. Both diseases involve psychotic symptoms. SCA17 is caused by an expanded polyglutamine tract in the TATA box-binding protein (*TBP*) gene. In the present study, we investigated the association between schizophrenia and CAG repeat length in common *TBP* alleles with fewer than 42 CAG repeats in a Japanese population (326 patients with schizophrenia and 116 healthy controls). We found that higher frequency of alleles with greater than 35 CAG repeats in patients with schizophrenia compared with that in controls ( $p = 0.042$ ). We also examined the correlation between CAG repeats length and age at onset of schizophrenia. We observed a negative correlation between the number of CAG repeats in the chromosome with longer CAG repeats out of two chromosomes and age at onset of schizophrenia ( $p = 0.020$ ). We further provided evidence that *TBP* genotypes with greater than 35 CAG repeats, which were enriched in patients with schizophrenia, were significantly associated with hypoactivation of the prefrontal cortex measured by near-infrared spectroscopy during the tower of Hanoi, a task of executive function (right PFC;  $p = 0.015$ , left PFC;  $p = 0.010$ ). These findings suggest possible associations of the genetic

variations of the *TBP* gene with risk for schizophrenia, age at onset and prefrontal function.

Keywords: Age at onset, near-infrared spectroscopy (NIRS), schizophrenia, TATA box-binding protein (*TBP*), tower of Hanoi

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Schizophrenia (MIM181500) is a common polygenic disease with a lifetime morbidity rate of approximately 1% across distinct populations. This disease is characterized by positive symptoms such as delusions and hallucinations, negative symptoms such as social withdrawal and blunted affect, and cognitive impairments. As studies in patients with schizophrenia have brought observations supporting a cerebellar impairment (cognitive dysmetria), disruption in the cortical-thalamic-cerebellar-cortical circuit (CCTCC) has been proposed as a possible pathophysiology of schizophrenia. (Andreassen *et al.* 1999; Mouchet-Mages *et al.* 2007).

Spinocerebellar ataxia (SCA) is a heterogeneous group of progressive brain diseases characterized by incoordination of movement and degeneration of cerebellum and/or brain stem. SCA is caused by pathological cytosine-adenine-guanine (CAG) trinucleotide repeat expansions in the mutated genes. Spinocerebellar ataxia type 17 (SCA17: MIN607136) is a rare autosomal dominant polyglutamine disease caused by expanded CAG/CAA (abbreviated as CAG) repeats in the gene encoding TATA box-binding protein (*TBP*), a general transcription initiation factor (Koide *et al.* 1999; Nakamura *et al.* 2001). This disease is characterized by various phenotypes, such as ataxia, nystagmus, cognitive impairment, and/or psychotic symptoms often diagnosed as a variety of psychiatric disorders, such as schizophrenia, bipolar disorder, depression, personality change, and/or dementia, before the onset of neurological symptoms (Bauer *et al.* 2004; Filla *et al.* 2002; Rolfs *et al.* 2003). The *TBP* gene is located on 6q27. The currently defined ranges of the CAG repeats in the *TBP* gene are as follows: normal; below 42, reduced penetrance; 43 to 48, full penetrance; above 49 repeats (Koide *et al.* 1999; Silveira *et al.* 2002). As abnormal expanded CAG repeats in the *TBP* gene cause SCA17 with a variety of psychotic symptoms, the *TBP* gene has been investigated as a candidate gene for psychoses. Expansion of the CAG repeat polymorphism in the *TBP* gene to greater than 43 CAGs has been found in patients with schizophrenia (Chen *et al.* 2005). On the other hand, no CAG repeat length greater than 43 in the *TBP* gene has



been reported in control subjects (Chen *et al.* 2005; Jones *et al.* 1997; Rubinsztein *et al.* 1996). However, only few patients with schizophrenia out of approximately thousands of chromosomes had expansion of the CAG repeat more than 43, suggesting that expansion of CAG repeats does not play a major role in the pathogenesis of schizophrenia (Chen *et al.* 2005; Jones *et al.* 1997; Rubinsztein *et al.* 1996). Thus, we hypothesized that common CAG repeat lengths in the *TBP* gene, below 42 CAGs, might be associated with the pathogenesis of schizophrenia. In this study, we firstly examined the possible association of CAG repeat length in the *TBP* gene with risk for schizophrenia.

An inverse correlation between CAG repeat length in the *TBP* gene and age at onset has been reported in SCA17 (van Roon-Mom *et al.* 2005). Gray matter volume reduction in the frontal lobe in SCA17 was correlated with scores of global assessment of functioning (GAF) scale measuring psychological, social and occupational levels (Lasek *et al.* 2006). Negative correlation between brain atrophy in the frontal lobe and degree of personality change has been reported in SCA17 (Lasek *et al.* 2006). Patients with SCA17 showed increased error rates in antisaccades and memory-guided saccades, which are implicated in a deficit of frontal inhibitory function (Hubner *et al.* 2007). These data suggested structural and functional abnormalities in the frontal lobe in SCA17. Therefore, we secondary examined whether the CAG repeat length in the *TBP* gene was associated with age at onset and with frontal lobe function in patients with schizophrenia.

## Materials and methods

### Subjects

A total of 442 individuals participated in our study (51.8% males, mean age  $\pm$  SD; 46.5  $\pm$  15.9 years). The subjects for this study were 326 unrelated patients with schizophrenia including 10 patients with schizoaffective disorder (50.3% males, mean age  $\pm$  SD; 51.6  $\pm$  14.5 years) and 116 unrelated healthy controls (55.2% males, mean age  $\pm$  SD; 31.9  $\pm$  9.9 years). The mean age differed significantly between groups ( $z = -11.6, p < 0.05$ , Mann-Whitney *U*-test), however, the sex ratio did not differ significantly between groups ( $\chi^2 = 0.8, df = 1, p = 0.37$ ). All subjects were biologically unrelated Japanese. Cases were recruited at Osaka University Hospital (155 patients, 41.9% males, mean age  $\pm$  SD; 43.6  $\pm$  13.1 years, outpatients/inpatients 106/49) and the related private psychiatric hospitals (171 patients, 57.9% males, mean age  $\pm$  SD; 58.9  $\pm$  11.4 years, outpatients/inpatients 0/171). Each schizophrenic research subject had received a diagnosis and assessment by at least two trained psychiatrists as a part of routine clinical diagnosis and treatment at university hospital and the related facilities, according to the Diagnostic and Statistical Manual of Mental Disorders, fourth edition (DSM-IV) criteria based on unstructured clinical interview and other available information including medical records and other research assessments. No patient was diagnosed on the basis of medical records alone. Controls, including hospital and institutional staffs, were recruited through local advertisements from Osaka. Psychiatrically, medically and neurologically healthy controls were evaluated using the structured clinical interview for DSM-IV-Non-Patient to exclude individuals who had received psychiatric medication. We also exclude the control subjects who were neurological disorders or first- or second-degree relatives with psychiatric disorders using an unstructured interview. We obtained

data of age at onset and duration of illness in 100 of 326 patients and of chlorpromazine equivalents of total antipsychotics in 155 of 326 patients from medical records or clinical interviews. The mean values  $\pm$  SD of age at onset, duration of illness and chlorpromazine equivalents of total antipsychotics were 25.8  $\pm$  10.5 years, 18.7  $\pm$  12.1 years and 511.2  $\pm$  466.2 mg/day. We obtained family history information about patients who had a first- or second-degree relative with schizophrenia or SCA. Twenty one of 99 patients (21.2%) had a relative with schizophrenia and one of 99 had a relative with undefined familial SCA other than SCA17 (Ohi *et al.* 2008). We have attempted to examine a general intelligence IQ test [full-scale Wechsler Adult Intelligence Scale (WAIS) revised or third edition] and Near-infrared spectroscopy (NIRS) of all subjects and positive and negative syndrome scale (PANSS) of all patients as much as we could. The patients recruited at the university hospital consisted of subjects who agreed blood drawing (47.7%, 74/155), blood drawing and receiving WAIS (38.7%, 60/155), blood drawing and receiving NIRS (5.8%, 9/155) and receiving all the examinations (7.7%, 12/155). As we failed to achieve the system to perform NIRS and WAIS in addition to blood drawing in the related private psychiatric hospitals, all patients who were recruited at the related hospitals agreed only blood drawing (100%, 171/171). The controls consisted of the subjects who agreed blood drawing (4.3%, 5/116), blood drawing and receiving WAIS (44.0%, 51/116) and receiving all the examinations (51.7%, 60/116). The reason why the subjects receiving NIRS were too small number is that the NIRS started in the middle of the overall study. The subjects who received WAIS and NIRS met the following additional criteria: (1) recruited at a single-institution, Osaka University, (2) excluded if they had neurological or medical conditions that could potentially affect the central nervous system, such as atypical headache, head trauma with loss of consciousness, chronic lung disease, kidney disease, chronic hepatic disease, thyroid disease, cancer with active stage, cerebrovascular disease, epilepsy or seizures, (3) excluded schizoaffective disorder and schizophrenia with comorbidity of substance-related disorder or mental retardation. Written informed consent was obtained from all subjects after the procedures had been fully explained. This study was carried out in accordance with the World Medical Association's Declaration of Helsinki and was approved by the Research Ethical Committee of Osaka University.

### NIRS analysis of activation of the prefrontal cortex

Activation of the prefrontal cortex (PFC) during the two tasks, the letter version of the verbal fluency test (VFT-letter) and the tower of Hanoi (TOH) measuring executive function, was analyzed in subjects who agreed to receive these tests using NIRS (Ikezawa *et al.* 2009). All subjects sat on a comfortable chair in a silent room and were instructed not to move during measurement. All tasks consisted of a pre-task period (30 seconds), a task period (60 seconds) and a post-task period (60 seconds).

In the VFT-letter, subjects were instructed to generate as many nouns as possible that start with a Japanese *hiragana* letter ('a' 'ka' 'sa' each for 20 seconds). They were also instructed to pronounce the syllables 'a', 'i', 'u', 'e', 'o' repeatedly during the pre-task and post-task periods. The total number of generated words was defined as task performance. This procedure was based on one used in a previous study (Kubota *et al.* 2005).

The TOH consisted of three pegs of equal size and several discs of different sizes. Subjects were instructed to follow three rules during the task. First, only one disc could be moved at a time. Second, a smaller disc must be placed on a larger disc. Third, it was prohibited to place discs anywhere other than on a peg. Following these rules, subjects were required to transfer the piled discs to another peg to form the original shape in as few moves as possible. To remove the activation elicited by the hand motion, they were instructed to move the smallest disc repeatedly during the pre-task and post-task periods. Before the measurement, they were asked to perform the 3-disc version of the TOH to confirm their comprehension of the instructions. In the 5-disc version of the TOH, the fewest moves required to achieve the goal is 31. The total number of effective moves was defined as task performance. This procedure was based on one used in a previous study (Gimenez *et al.* 2003).

NIRS measurements were conducted using a two-channel system (NIRO-200, Hamamatsu Photonics, Japan). The NIRO-200 utilizes three kinds of near-infrared light (775 nm, 810 nm and 850 nm) and could detect changes in the concentration of oxygenated hemoglobin ([oxyHb]) and deoxygenated hemoglobin ([deoxyHb]) in primal venous blood in the cerebral cortex. Two pairs of emission (light source: laser-diode) and detection probes (light detector: photo-diode) were attached to the bilateral foreheads of the subjects. One detection probe was located in the Fp1 and the corresponding emission probe was located approximately in F7, while the other pair of probes was located in Fp2-F8, according to the international 10/20 system for electroencephalography. The distance between the paired probes was 3 cm. This probe setting enabled us to detect the hemodynamic changes in the bilateral prefrontal cortical areas corresponding to the superior frontal gyrus and the inferior frontal gyrus (Okamoto *et al.* 2004). The sampling rate for measurements of signals was 6 Hz. The path length factor was defined as 24 cm. The scale of quantity changes of the hemoglobin concentration was  $\mu\text{mol/l}$ .

In the data analysis, changes in [oxyHb] were used since [oxyHb] is sensitive to changes in regional cerebral blood volume (rCBV) (Hoshi 2003; Hoshi *et al.* 2001). The mean level of [oxyHb] during the last 10 seconds of the pre-task period was defined as baseline. Excluding the first 10 seconds of the task period, the mean levels of [oxyHb] for the remaining 50 seconds of the task period in VFT-letter and TOH tasks were defined as the activation levels. The difference between activation and baseline levels was defined as the size of activation ( $\Delta$  [oxyHb]).

### Genetic association analysis

Blood was collected from subjects and genomic DNA was extracted from whole blood according to standard procedures. The genomic segment of the CAG repeats and the flanking regions were amplified by polymerase chain reaction (PCR) using a pair of primers described in a previous study (Koide *et al.* 1999), 5'-GACCCACAGCCTATTGCA-3' and 5'-TTGACTGCTGAACGGGTGCA-3'. PCR for each sample was carried out in a total volume of 40  $\mu\text{l}$  using a Gene Amp<sup>®</sup> PCR System 9700 (Applied Biosystems, CA, U.S.A.). The PCR cycling conditions were 94°C for 10 min, 30 cycles of 94°C for 1 min, 60°C for 1 min and 72°C for 1 min, followed by incubation at 72°C for 10 min. PCR products were purified using a QIA quick<sup>®</sup> PCR Purification Kit (QIAGEN, CA, U.S.A.) and purification products were sequenced using a Big Dye<sup>®</sup> Terminator v1.1 Cycle Sequencing Kit (Applied Biosystems, CA, U.S.A.). Cycle sequencing conditions were 96°C for 2 min, and 25 cycles of 96°C for 20 seconds, 50°C for 30 seconds and 60°C for 2 min, using a Gene Amp<sup>®</sup> PCR System 9700. PCR products from cycle sequencing were purified using a Big Dye<sup>®</sup> XTerminator<sup>™</sup> Purification Kit (Applied Biosystems, CA, U.S.A.) and they were sequenced using an ABI PRISM<sup>®</sup> 3700 DNA Analyzer (Applied Biosystems, CA, U.S.A.). Sequence analysis was performed with SEQUENCHER ver. 4.7 (Gene Codes, U.S.A.). To check for the reliability of the sequencing method by Koide *et al.* (1999), we performed the direct DNA sequencing in 48 randomly selected subjects (96 chromosomes) twice. The sequencing data of individual subjects were in agreement with each other.

### Statistical analysis

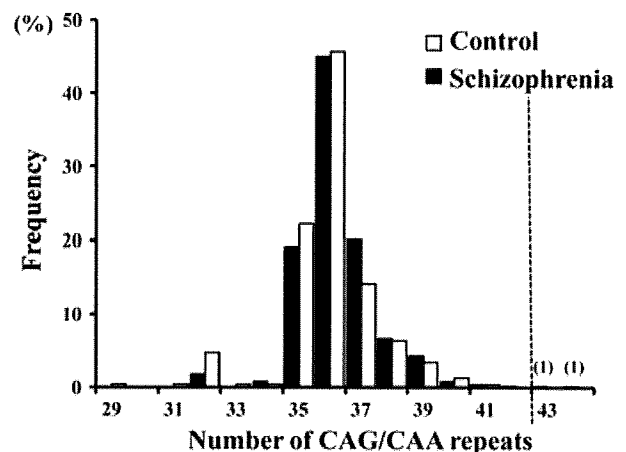
Statistical analysis was performed using SPSS 12.0J for Windows (SPSS Japan Inc., Tokyo, Japan). Differences in clinical characteristics between patients and controls were analyzed using  $\chi^2$  tests for categorical variables and Mann-Whitney *U*-test for continuous variables. A repeat length below 35 CAGs has been generally considered to be stable, producing no symptoms in several polyglutamine diseases, while a repeat length above 36 CAGs has been considered to be unstable because it causes disease symptoms (Trottier *et al.* 1995; van Roon-Mom *et al.* 2005). Thus, we divided all subjects into two allele groups (S for short  $\leq 35$  and L for long  $> 35$  alleles) based on a split at 35 CAG repeats in the *TBP* gene. As there were few individuals with S/S homozygote (patients; S/S 2: S/L 141: L/L 183, controls; S/S 0: S/L 66: L/L 50), we divided the participants

into two groups (individuals with S/S or S/L genotypes, those with L/L genotypes) (Tables 1 and 2). In genetic association analysis, the difference of the mean alleles between patients and controls was analyzed by Mann-Whitney *U*-test. The difference of frequencies in individuals with S or L allele in the *TBP* gene between patients and controls was analyzed by  $\chi^2$  test. We analyzed the difference in age at onset in patients between sex or *TBP* genotypes (S/L or L/L genotype) by Mann-Whitney *U*-test. We investigated correlations between age at onset in patients and the number of CAG repeats in the chromosome with longer CAG repeats out of two chromosomes. When the subjects were homozygotes of the CAG repeat, we treated the CAG repeat as longer one. Multiple linear regression analysis was performed to explore determinants of age at onset in patients with schizophrenia. Biologically plausible predictions (sex; male: 0, female: 1) and the number of CAG repeats were included in this model. In NIRS analysis, the effects of the *TBP* genotype and diagnosis on frontal cortex function were analyzed by a two-way ANCOVA with age, sex and education years as covariates. We performed Bonferroni correction for solving the problems of multiple testing. The correction was performed based on independent four tests (bilateral PFC, TOH and VFT-letter). Two-tailed *p* values were used for all tests. Statistical significance was set at  $< 0.05$ .

## Results

### Genetic association analysis

We examined the possible association between schizophrenia and CAG repeat length in the *TBP* gene with fewer than 42 CAG repeats. The distribution of *TBP* allele frequencies between patients and controls is shown in Fig. 1. The CAG repeats in the *TBP* gene is highly polymorphic in both patients and controls in a Japanese population, similar to other populations reported previously (Chen *et al.* 2005; Rubinsztein *et al.* 1996; Wu *et al.* 2005). The distribution of



**Figure 1: Distribution of *TBP* allele frequencies based on CAG repeat length in patients with schizophrenia and controls.** The closed bar denotes individuals with schizophrenia. The open bar denotes control individuals. Y axis: percentage of the each CAG repeats in the *TBP* gene. X axis: number of CAG repeats in the *TBP* gene. The dotted line is the boundary line of expanded CAG repeats in the *TBP* gene between patients with SCA17 and controls. There were two patients with schizophrenia above 43 CAG repeats.

alleles showed significant deviation from HWE in the control group ( $p < 0.0001$ ). Allele lengths in patients ranged from 29 to 44 trinucleotide repeats, while those in controls ranged from 29 to 41 trinucleotide repeats. Although the mean allele length was significantly longer in patients compared with controls ( $36.2 \pm 1.4$  repeats for patients and  $35.9 \pm 1.5$  repeats for controls;  $z = -2.5, p = 0.013$ , Mann–Whitney  $U$ -test), the most common allele was 36 in both patients and controls and the allele distributions were not likely to be significantly different between the patients and the controls. We revealed significantly lower S allele frequency in patients compared with that in controls in the analysis based on the split at 35 CAG repeats (patients vs. controls: 22.2% vs. 28.9%,  $p = 0.042$ , odds ratio = 0.70, 95% confidence interval = 0.50–0.99). General demographic information of subjects for the genetic association analysis is shown in Table 1. Demographic variables did not significantly differ in age, sex or full-scale IQ between two *TBP* genotype groups (S carriers and L/L homozygotes) in controls. There was no difference between *TBP* genotype groups in demographic variables, age, sex, duration of illness, family history of schizophrenia, PANSS scores, full-scale IQ or doses of antipsychotics in patients with schizophrenia. SCA17 and schizophrenia have strong familial components, however, our results in this cohort would not be related to the family history of schizophrenia because of no difference in the family history. Abnormal expansions above 43 CAG repeats in the *TBP* gene were found in two patients with schizophrenia (43 and 44 repeats). The expanded sequence alignments were (CAG)3(CAA)3 (CAG)6 (CAA)(CAG)(CAA) (CAG)27 (CAA)(CAG) with a total

of 44 repeats and (CAG)3(CAA)3 (CAG)8 (CAA)(CAG)(CAA) (CAG)24 (CAA)(CAG) with a total of 43 repeats.

### Correlation of age at onset of schizophrenia with CAG repeats length in the *TBP* gene

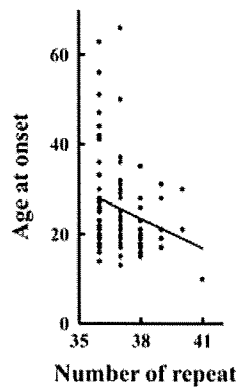
We investigated the association between age at onset of schizophrenia and *TBP* genotype based on a split at 35 CAG repeats. We found marginally earlier age at onset of schizophrenia in individuals with L/L genotype compared to those with S-carriers (S-carriers;  $n = 45$ , mean  $\pm$  SD,  $28.9 \pm 13.5$  years, range 13–66 years, individuals with L/L genotype;  $n = 55$ , mean  $\pm$  SD,  $22.9 \pm 6.5$  years, range 10–41 years,  $z = -1.8, p = 0.07$ ). We further examined the correlation between age at onset of schizophrenia and the number of CAG repeats in the chromosome with longer CAG repeats out of two chromosomes, because earlier onset of disease has been reported in SCA17 patients with expanded CAG repeats in the *TBP* gene (van Roon-Mom *et al.* 2005). There was an inverse correlation between age at onset of schizophrenia and the number of CAG repeats in the chromosome with longer CAG repeats out of two chromosomes, as shown in Fig. 2 ( $r = -0.23, df = 98, p = 0.020$ ). On the other hand, the number of CAG repeats in the chromosome with shorter CAG repeats was not correlated with age at onset of schizophrenia ( $r = 0.11, df = 98, p = 0.28$ ). It is well known that male individuals develop schizophrenia at an earlier age than female (Hafner 2003; Takahashi *et al.* 2000). As the difference in age at onset between male and female patients with schizophrenia was marginal in our cohort (male;  $23.5 \pm 8.6$ , female;  $27.5 \pm 11.9, z = -1.9, p = 0.06$ ),

**Table 1:** General demographic information of subjects for the genetic association analysis

Variables	Patients		Controls	
	S/S, S/L ( $n = 143$ )	L/L ( $n = 183$ )	S/L ( $n = 66$ )	L/L ( $n = 50$ )
Age (years)	$52.4 \pm 13.0$	$51.0 \pm 15.5$	$31.8 \pm 10.0$	$31.9 \pm 9.9$
Sex (Male/Female)	72/71	92/91	32/34	32/18
Age at onset (years)	$28.9 \pm 13.5$	$22.9 \pm 6.5$	—	—
Duration of illness (years)	$18.5 \pm 13.9$ ( $n = 45$ )	$18.8 \pm 10.7$ ( $n = 55$ )	—	—
Family history of schizophrenia (yes/no)	7/43 ( $n = 50$ )	14/56 ( $n = 70$ )	—	—
Positive symptoms <sup>†</sup>	$16.3 \pm 5.1$	$16.9 \pm 6.7$	—	—
Negative symptoms <sup>†</sup>	$16.9 \pm 6.3$ ( $n = 46$ )	$17.5 \pm 7.1$ ( $n = 57$ )	—	—
CPZeq of total antipsychotics (mg/day)	$527.9 \pm 460.9$ ( $n = 64$ )	$499.5 \pm 412.0$ ( $n = 91$ )	—	—
Full scale IQ	$88.7 \pm 16.6$ ( $n = 30$ )	$87.6 \pm 18.7$ ( $n = 42$ )	$108.5 \pm 11.5$ ( $n = 63$ )	$109.4 \pm 13.3$ ( $n = 48$ )

Means  $\pm$  SD are shown.

<sup>†</sup>PANSS, Positive and Negative Syndrome Scale. CPZeq: Chlorpromazine equivalents; S: short allele; less than 35 repeats, L: long allele; more than 36 repeats. As there were few individuals with S/S homozygote (patients; S/S 2: S/L 141: L/L 183, controls; S/S 0: S/L 66: L/L 50), we divided participants into two groups (S/S or S/L genotype group, L/L genotype group).



**Figure 2: Correlation between the number of CAG repeats in longer TBP alleles and the age at onset of schizophrenia.**

A significantly negative correlation between the age at onset for schizophrenia (y axis) and the number of CAG repeats in the TBP gene (x axis) is observed in patients with schizophrenia ( $n = 100$ ). The regression line is represented by:  $y = -1.6x + 84.8$ . Each patient with schizophrenia is plotted as a closed circle.

we performed secondary multiple linear regression analysis with age at onset as a dependent variable and the number of CAG repeats in the chromosome with longer CAG repeats out of two chromosomes and sex as independent variables. The larger number of CAG repeats in the chromosome with longer CAG repeats ( $\beta = -0.24, p = 0.016$ ) and male ( $\beta = -0.19, p = 0.049$ ) significantly predicted earlier age at onset for schizophrenia [ $r$  (correlation coefficient) = 0.30,  $r^2$  (coefficient of determination) = 0.09,  $df = 98, p = 0.010$ ].

**Frontal lobe function**

We examined a possible association between TBP genotype and frontal lobe function, such as executive function and verbal fluency, in patients with schizophrenia and controls. Demographic variables did not significantly differ in age or sex, except for education years (cases;  $14.0 \pm 2.2$  controls,  $15.5 \pm 2.0, z = -2.6, p = 0.010$ ), between patients with schizophrenia and controls. There was no difference in demographic variables, age, sex or education years between

two TBP genotype groups (individuals with S/L heterozygote and individuals with L/L homozygote) in the total subjects. In controls, demographic variables did not significantly differ in age, sex or education years between two TBP genotype groups. In patients with schizophrenia, there was no difference in demographic variables, sex, education years or doses of antipsychotics, except for age ( $z = -2.0, p = 0.043$ ), between TBP genotype groups (Table 2). However, no correlation between age and activation of the PFC was found in patients (right:  $r = -0.34, df = 19, p = 0.14$ , left:  $r = -0.34, df = 19, p = 0.14$ ). Two-way ANCOVA on activation of PFC during the TOH by NIRS revealed significant effects of diagnosis (right PFC:  $F_{1,74} = 5.1, p = 0.027$ , left PFC:  $F_{1,74} = 8.1, p = 0.0058$ ) and genotype (right PFC:  $F_{1,74} = 6.2, p = 0.015$ , left PFC:  $F_{1,74} = 7.0, p = 0.010$ ) (Table 3, Fig. 3). No genotype-diagnosis interaction was found in the right or the left PFC (right PFC:  $F_{1,74} = 0.1, p = 0.76$ , left PFC:  $F_{1,74} = 0.5, p = 0.48$ ). The association remained significant in both diagnosis ( $p = 0.023$ : corrected) and genotype ( $p = 0.040$ : corrected) effects on the activation of the left PFC, even after a Bonferroni correction was applied. Individuals with the L/L genotype, which was enriched in patients with schizophrenia, showed lower activation of the PFC during the TOH than those with S/L genotype (Fig. 3). There was no effect of diagnosis ( $F_{1,74} = 2.1, p = 0.15$ ), genotype ( $F_{1,74} = 0.1, p = 0.74$ ) or genotype-diagnosis interaction ( $F_{1,74} = 0.1, p = 0.80$ ) on the performance of the TOH task (Table 3). We did not find the effect of genotype, diagnosis, or genotype-diagnosis interaction on the activation of the PFC during the VFT-letter task or on the performance of the VFT-letter task, except for the effect of diagnosis on the performance of VFT-letter ( $F_{1,74} = 16.3; p = 0.00014, corrected p = 0.00056$ ) (Table 3).

**Discussion**

We report a possible association between schizophrenia and CAG repeat length in the TBP gene. A significantly higher frequency of TBP alleles with more than 36 CAG repeats was observed in patients with schizophrenia than that in controls. Dentatorubral-pallidoluyisian atrophy, Huntington

**Table 2: Demographic variable scores for subjects included in the NIRS analysis**

Variables	Patients		Controls	
	S/L ( $n = 10$ )	L/L ( $n = 11$ )	S/L ( $n = 34$ )	L/L ( $n = 26$ )
Age (years)	$41.0 \pm 12.1$	$30.1 \pm 10.1^*$	$36.2 \pm 11.2$	$33.5 \pm 10.0$
Age at onset (years)	$25.9 \pm 8.8$	$19.9 \pm 5.9$	—	—
Sex (Male/Female)	8/2	4/7	17/17	14/12
Education (years)	$14.6 \pm 1.8$	$14.0 \pm 2.4$	$15.2 \pm 2.3$	$16.0 \pm 1.3$
CPZeq of total antipsychotics, (mg/day)	$653.3 \pm 372.7$	$522.7 \pm 492.0$	—	—

Means  $\pm$  SD are shown. CPZeq: Chlorpromazine equivalents; S: short allele; less than 35 repeats, L: long allele; more than 36 repeats. There was no individual with S/S homozygote in the NIRS analysis.

\* $p < 0.05$ , compared with individuals with S/L genotype.

**Table 3:** Effects of *TBP* genotype and diagnosis on activation of the PFC measured by NIRS during the TOH and VFT-letter tasks

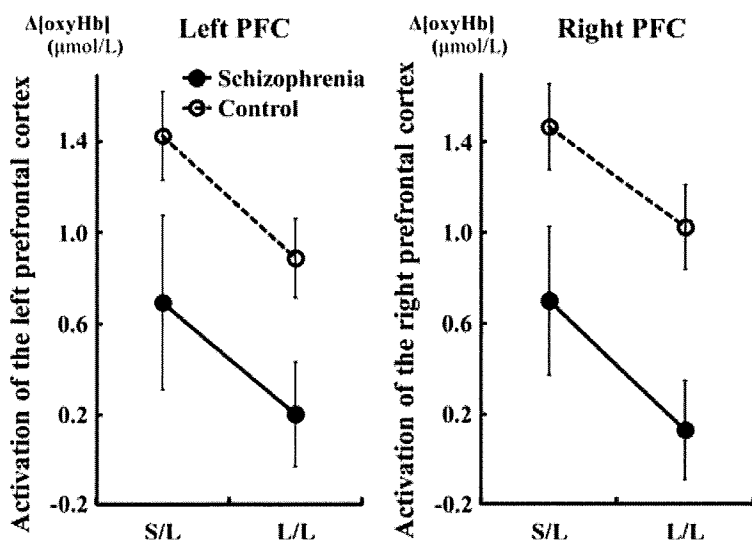
	Patients		Controls		<i>F</i> <sub>1,74</sub> values (ANCOVA)		
	S/L	L/L	S/L	L/L	Diagnosis effect	Genotype effect	Interaction
<b>TOH</b>							
Activation of the rt. PFC <sup>†</sup>	0.70 ± 1.23	0.20 ± 0.77	1.44 ± 1.16	0.90 ± 0.89	5.1*	6.2*	0.1
Activation of the lt. PFC <sup>†</sup>	0.69 ± 1.02	0.12 ± 0.72	1.44 ± 1.09	1.01 ± 0.94	8.1**	7.0*	0.5
Performance score	7.8 ± 6.6	9.1 ± 5.0	11.7 ± 6.1	11.4 ± 6.1	2.1	0.1	0.1
<b>VFT-letter</b>							
Activation of the rt. PFC <sup>†</sup>	1.29 ± 2.70	0.71 ± 0.80	1.23 ± 1.06	0.92 ± 1.64	0.0	2.0	0.3
Activation of the lt. PFC <sup>†</sup>	1.18 ± 2.88	0.62 ± 0.83	1.19 ± 0.93	1.00 ± 1.25	0.1	1.6	0.3
Performance score	10.3 ± 4.2	14.5 ± 3.6	16.6 ± 3.9	16.8 ± 4.4	16.3**	3.4	1.7

PFC, prefrontal cortex; NIRS, near-infrared spectroscopy; TOH, tower of Hanoi, VFT-letter, verbal fluency test-letter, <sup>†</sup>The scale of quantity changes of the hemoglobin concentration was μ mol/l. Means ± SD are shown. \**p* < 0.05, \*\**p* < 0.01

disease and various subtypes of SCA are caused by expanded CAG repeats encoding a polyglutamine chain. Although the mechanism underlying these diseases and CAG repeat length remains to be elucidated, it has been assumed to involve a gain/loss of function. SCA17 is caused by expanded CAG repeats in the *TBP* gene (no penetrance: below 42; reduced penetrance: 43 to 48; full penetrance: above 49 repeats) (Koide *et al.* 1999; Silveira *et al.* 2002). More than 36 CAG repeats have been considered to be unstable and to cause the symptoms of the other polyglutamine diseases. On the other hand, fewer than 35 CAG repeats are stable and are not related to the development of the other polyglutamine diseases. (Trottier *et al.* 1995; van Roon-Mom *et al.* 2005). The threshold of CAG repeats in SCA17 is higher compared with the threshold of CAG repeats in other CAG repeat diseases (Pearson *et al.* 2005; van Roon-Mom *et al.* 2005). Therefore, the range of no penetrance of CAG repeats in the *TBP* gene includes unstable CAG repeats from 36 to 42 in SCA17 patients. Although the unstable repeats in the *TBP* gene may not play a role in the pathogenesis of SCA17, there

is a possibility that the unstable CAG repeats are involved in the pathogenesis of schizophrenia. In the present study, CAG repeats longer than 43 were 2/652 alleles in patients with schizophrenia and 0/232 alleles in controls. Consistent with the previous studies, our result suggests that the CAG repeats longer than 43 CAGs in the *TBP* gene might not play a major role in the pathogenesis of schizophrenia (Chen *et al.* 2005; Rubinsztein *et al.* 1996) (Supporting information Table S1). Limitations of this analysis are the small size of the control population compared with the patient population and the possibilities of ethnic heterogeneity in CAG repeat length in the *TBP* gene.

We demonstrated a reverse correlation between age at onset of schizophrenia and longer CAG repeats length in the *TBP* gene. Correlation between CAG repeat length and earlier age at onset or severity of symptoms has been frequently reported in polyglutamine diseases including SCA17 (Nakamura *et al.* 2001; Rolfs *et al.* 2003; Walker 2007). Anticipation is a phenomenon whereby the symptoms of a genetic disorder become apparent at an earlier age



**Figure 3:** Effect of *TBP* genotype and diagnosis on activation of the PFC measured by NIRS during the tower of Hanoi task. Individuals homozygous for the L allele in the *TBP* gene, which was enriched in patients with schizophrenia, showed lower activation of the PFC compared to those with S/L heterozygote (schizophrenia; S/L *n* = 10, L/L *n* = 11, control; S/L *n* = 34, L/L *n* = 26). Closed circles represent subjects with schizophrenia. Open circles represent healthy controls. Bars represent the standard error. PFC, prefrontal cortex. S: short allele; less than 35 repeats, L: long allele; more than 36 repeats.

at onset as it is passed on to the next generation. Anticipation is caused by extent of the CAG repeats during paternal meiosis. CAA interpretation in the expanded CAG repeat at the *TBP* locus stabilizes the repeat tract during intergenerational transmission (Maltecca *et al.* 2003). Anticipation has been observed not only in a variety of SCAs, but also in schizophrenia (McInnis *et al.* 1999). We could not investigate anticipation and paternal/maternal transmission of the CAG/CAA repeats in patients with schizophrenia, because we could not obtain the DNA from parents of patients with schizophrenia.

Impairment of executive function has been reported in patients with schizophrenia (Fioravanti *et al.* 2005; Shad *et al.* 2006). Lower performance of the TOH, which measures executive function related to frontal lobe function, has been reported in patients with schizophrenia (Bustini *et al.* 1999; Schuepbach *et al.* 2002). We found lower activation of the PFC measured by NIRS during the TOH task in patients with schizophrenia compared with that in controls (diagnosis effect). Moreover, hypoactivation of the PFC during the TOH was also revealed in individuals homozygous for the L allele, which was enriched in patients with schizophrenia compared with controls, compared to those with S/L heterozygote (genotype effect). Our results suggest the impact of the TBP genotype on prefrontal physiological activation. In the NIRS analysis, significant age difference was observed between genotype groups in patients. Although age-dependent decreases in task-related cerebral oxygenation responses have been demonstrated in NIRS measurements (Mehagnoul-Schipper *et al.* 2002), age did not correlate with activation of the PFC in our study. The basic limitations of this paper are that the clinical ascertainment was unstructured as the NIRS only began half way through the collection of samples. With such an approach, it is difficult to eliminate different systematic biases within the different individual studies that comprise the paper. Further studies are needed because of the relatively small sample size in this study.

In conclusion, we indicated a novel concept that sub-clinical expansion above 36 CAG repeat within the *TBP* gene might be associated with the risk for schizophrenia, age at onset and prefrontal function. Further studies are needed to investigate the association between CAG repeat length in the *TBP* gene and length-affected function in patients with schizophrenia.

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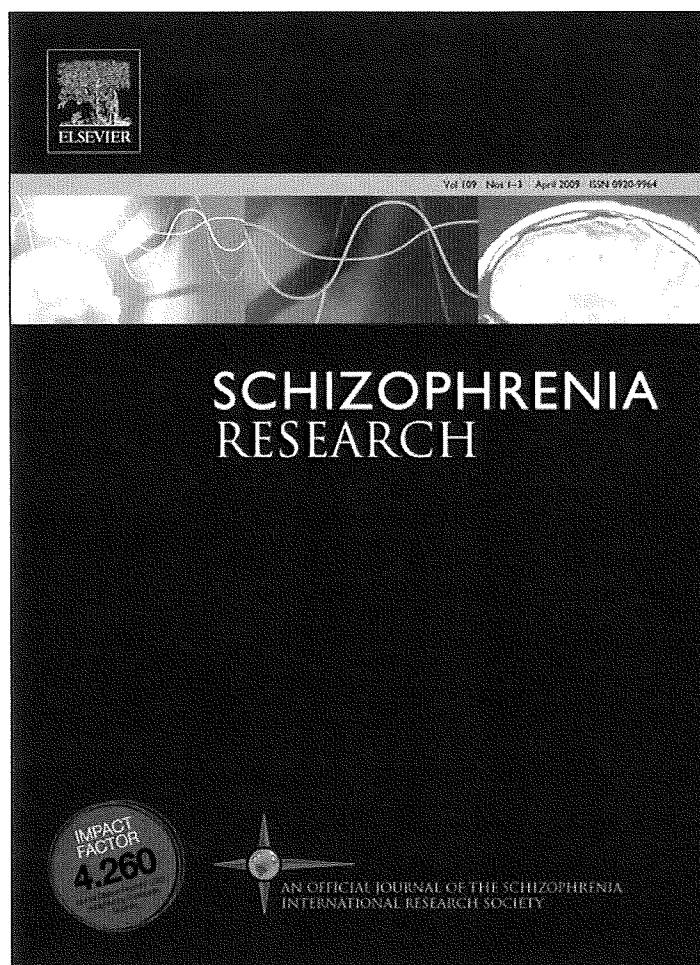
## Supporting Information

Additional Supporting Information may be found in the online version of this article:

**Table S1:** Comparison of total alleles, modal allele and allele range among the present study and previous studies

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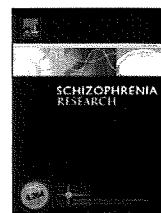
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## Association study of the *G72* gene with schizophrenia in a Japanese population: A multicenter study

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### ABSTRACT

*G72* is one of the most widely tested genes for association with schizophrenia. As *G72* activates the D-amino acid oxidase (DAO), *G72* is termed D-amino acid oxidase activator (DAOA). The aim of this study is to investigate the association between *G72* and schizophrenia in a Japanese population, using the largest sample size to date (1774 patients with schizophrenia and 2092 healthy controls). We examined eight single nucleotide polymorphisms (SNPs), which had been associated with schizophrenia in previous studies. We found nominal evidence for association of alleles, M22/rs778293, M23/rs3918342 and M24/rs1421292, and the genotype of M22/rs778293 with schizophrenia, although there was no association of allele or genotype in the other five SNPs. We also found nominal haplotypic association, including M15/rs2391191 and M19/rs778294 with schizophrenia. However, these associations were no longer positive after correction for multiple testing. We conclude that *G72* might not play a major role in the risk for schizophrenia in the Japanese population.

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### 1. Introduction

Schizophrenia (MIM181500) is a common neuropsychiatric disorder affecting 0.5–1% of the general population worldwide. Family, twin, and adoption studies of schizophrenia have indicated that there is a strong genetic factor with an estimated heritability of approximately 80% (Cardno and Gottesman, 2000). Several genome-wide linkage scan

studies of whole-genome linkage scans show suggestive linkages to schizophrenia on chromosomes 1q, 3p, 5q, 6p, 8p, 11q, 13q, 14p, 20q and 22q (Owen et al., 2004). Chumakov et al. (2002) focused on chromosome region 13q22–q34, which have suggested by a number of linkage studies (Blouin et al., 1998; Brzustowicz et al., 1999; Chumakov et al., 2002; Lin et al., 1995). They built a map of 191 single nucleotide polymorphisms (SNPs) in a 5-Mb segment on 13q34 and found robust evidence for genetic association between schizophrenia and several SNPs in the narrowed 65-kb region. Two overlapping genes, *G72* (MIN 607408) and *G30* (MIN 607415), which are transcribed in opposite directions and span approximately 29 and 47 kb of genomic sequences, were annotated in this region (Chumakov et al., 2002). In vitro translation of these genes resulted in a product for *G72* only. Chumakov et al. (2002) demonstrated that the *G72* protein (i.e. LG72), which is only known in higher primates, acts as an activator of the DAO protein. The *G72* protein was therefore referred to as DAO activator (DAOA). Gene expression analysis of *G72* in postmortem dorsolateral prefrontal cortices showed a tendency toward increased expression of *G72* mRNA in schizophrenia than that in control (Korostishevsky et al., 2004), although the reported increase of *G72* expression has yet to be replicated. Furthermore, the activity of DAO was also increased in postmortem cortices from patients with schizophrenia (Madeira et al., 2008). D-serine is an agonist at the glycine modulation site of the N-methyl-D-aspartate (NMDA) -type glutamate receptor and plays a role in neuronal migration and cell death (Scolari and Acosta, 2007). As DAO oxidizes and degrades D-serine, DAO is considered to modulate NMDA function in cortex. Lower serum level of D-serine was revealed in patients with schizophrenia as compared to that in healthy controls. Furthermore, administration of D-serine as add-on medication reduced parts of the symptoms of schizophrenia (Boks et al., 2007). Chumakov et al. (2002) hypothesized that the activation of DAO activity by a *G72* protein product might promote degradation of D-serine and cause a hypofunction of glutamate-signaling through the NMDA receptor in schizophrenia. However, the potential relationship between *G72* and NMDA receptor system still lacks supporting evidence.

Significant associations of *G72* with schizophrenia have been reported in various populations other than Japanese, such as French Canadians, Russians, German, Palestinian Arabs, South African, Ashkenazic Jewish, Chinese, Taiwanese, Scottish, Korean and Irish (Addington et al., 2004; Chumakov et al., 2002; Corvin et al., 2007; Fallin et al., 2005; Hall et al., 2004; Hong et al., 2006; Korostishevsky et al., 2004, 2006; Ma et al., 2006; Schumacher et al., 2004; Shin et al., 2007; Shinkai et al., 2007; Wang et al., 2004; Yue et al., 2006, 2007; Zou et al., 2005). The majority of replication studies of *G72* have indicated significant associations of alleles, genotypes or haplotypes with schizophrenia. However, a minority have reported no association between *G72* and schizophrenia (Bakker et al., 2007; Goldberg et al., 2006; Liu et al., 2006; Mülle et al., 2005; Sanders et al., 2008; Vilella et al., 2008; Williams et al., 2006; Wood et al., 2007). Associations of this gene were also reported with bipolar disorder (Chen et al., 2004; Hattori et al., 2003; Prata et al., 2008; Schumacher et al., 2004; Williams et al., 2006), major depression

(Rietschel et al., 2008) and panic disorder (Schumacher et al., 2005).

In this study, we examined possible association between *G72* polymorphisms and schizophrenia in a large Japanese population.

## 2. Materials and methods

### 2.1. Subjects

The subjects for this study consisted of 1774 patients with schizophrenia [males: 55.5%, mean age of 45.6 years (SD 15.1)] and 2092 healthy controls [males: 49.3%, mean age of 45.0 years (SD 19.7)], which is the largest sample size to date for *G72* association study. There was no significant difference in age between patients and controls groups ( $P=0.30$ ), while the sex ratio differed significantly between groups ( $P=0.00014$ ). All subjects were biologically unrelated Japanese and were recruited at four geographic regions, which were located on the main islands in Japan: Osaka, Aichi, Tokushima and Tokyo. There is little possibility for great ethnic/genetic difference among these regions for feature of homogeneous race in Japan (Yamaguchi-Kabata et al., 2008). Cases were recruited from both outpatients and inpatients at university hospitals and related psychiatric facilities. Controls, including hospital and institutional staffs, were recruited from local advertisements. Each patient with schizophrenia had received a diagnosis and assessment by at least two trained psychiatrists as a part of routine clinical diagnosis and treatment at the university hospitals and the related psychiatric facilities, according to the Diagnostic and Statistical Manual of Mental Disorders, fourth edition (DSM-IV) criteria based on unstructured clinical interviews and other available information including medical records and other research assessments. No patient was diagnosed on the basis of medical records alone. Psychiatrically healthy controls were evaluated using unstructured interviews to exclude individuals who had received psychiatric medication. Written informed consent was obtained for all subjects after the procedures had been fully explained. This study was carried out in accordance with the World Medical Association's Declaration of Helsinki and approved by the Research Ethical Committee of Osaka University, Fujita Health University, Nagoya University, Tokushima University and Juntendo University.

### 2.2. SNP genotyping and genomic sequencing

Eight SNPs, rs3916965 (M12), rs3916967 (M14), rs2391191 (M15), rs778294 (M19), rs3916970 (M20), rs778293 (M22), rs3918342 (M23) and rs1421292 (M24), were selected from the genomic region of the *G72* gene and its flanking regions. The designations of the SNPs in parentheses are according to Chumakov et al. (2002). To examine the association between schizophrenia and previously associated SNPs in a Japanese cohort, we chose eight SNPs, which had been associated with schizophrenia in previous studies, although our study design using these SNPs does not provide complete *G72* gene coverage. The positions of the eight SNPs analyzed in the present study are indicated in Supplementary Fig. 1. Venous blood was collected from the subjects and genomic DNA was

**Table 1**  
SNP genotype and allele distribution in patients with schizophrenia and controls.

Marker position	M/m <sup>c</sup>	SCZ (%)			CON (%)			MAF		Genotypic		Allelic		OR
		SNP number <sup>a</sup>	Kb <sup>b</sup>	M/M	M/m	m/m	M/M	M/m	m/m	SCZ (%)	CON (%)	P-value <sup>d</sup> (df=2)	P-value <sup>d</sup> (df=1)	
M12	0	A/G	57.1	35.5	7.4	55.9	37.6	6.5	25.1	25.3	0.26	0.87	0.99	
M14	14	G/A	55.3	36.7	8.0	53.7	39.2	7.1	26.3	26.7	0.21	0.75	0.98	
M15	2	A/G	55.1	37.0	7.9	54.3	38.8	6.9	26.4	26.3	0.34	0.91	1.01	
M19	23	G/A	72.9	24.4	2.7	71.5	25.9	2.6	14.9	15.5	0.55	0.43	0.95	
M20	12	A/G	39.0	46.1	14.9	40.9	45.3	13.8	38.0	36.4	0.4	0.17	1.08	
M22	15	A/G	54.7	38.7	6.6	58.9	35.0	6.1	26.0	23.6	<b>0.034</b>	<b>0.019</b>	1.13	
M23	17	T/C	31.7	49.4	18.9	34.7	48.3	17.0	43.6	41.2	0.09	<b>0.030</b>	1.11	
M24	12	A/T	24.9	50.6	24.5	28.1	49.0	23.0	49.8	47.4	0.07	<b>0.037</b>	1.10	

SCZ, patients with schizophrenia; CON, healthy controls; m, minor allele; M, major allele; MAF, minor allele frequency; OR, odds ratio.

<sup>a</sup>The db SNP IDs equivalent to the M-SNP IDs designed by Chumakov et al. (2002) are the following: M12 (rs3916965), M14 (rs3916967), M15 (rs2391191), M19 (rs778294), M20 (rs3916970), M22 (rs778293), M23 (rs3918342), and M24 (rs1421292).

<sup>b</sup>Distances inter-SNPs are shown (Kb).

<sup>c</sup>The first shown alleles are major allele. All the alleles are represented according to the forward DNA sequence to make them comparable with the previous published data.

<sup>d</sup>Significant *P*-values (< 0.05) are in bold face.

extracted from whole blood according to standard procedures. Genotyping of the SNPs was carried out using TaqMan assays (Applied Biosystems, Foster City, California, USA). TaqMan probes and Universal PCR Master Mix were obtained from Applied Biosystems. A 5- $\mu$ l total reaction volume was used, and allelic-specific fluorescence was measured using an ABI PRISM 7900 Sequence Detector System (Applied Biosystems). In addition, we genotyped eight SNPs in 32 randomly selected subjects (64 chromosomes) by a direct DNA sequencing method to check for typing errors by the TaqMan method. We confirmed that all genotypes determined by the direct sequencing method were in agreement with the genotypes of the TaqMan methods for all eight SNPs. Detailed information on the PCR conditions and the primer pairs are shown in Supplementary Methods and Supplementary Table 1.

### 2.3. Statistical analysis

Statistical analysis was performed using SNPalyze V5.1.1 Pro software (DYNACOM, Yokohama, Japan). The presence of Hardy-Weinberg equilibrium (HWE) was examined by using the  $\chi^2$  test for goodness of fit. The statistical significance of HWE analysis was defined at *P* < 0.01. The allelic and genotypic distributions of G72 polymorphisms between patients and controls were analyzed using  $\chi^2$  tests for independence. We performed correction for multiple testing in single marker analysis by using the SNPSpD program (Nyholt, 2004). Pairwise linkage disequilibrium (LD) analysis, expressed by *D'* values, was applied to detect the intermarker relationship in each group using the Haploview software (<http://www.broad.mit.edu/mpg/haploview/contact.php>). Haplotype frequencies were estimated by the method of maximum likelihood from the genotyping data through the use of the Expectation-Maximization algorithm. Rare haplotypes found in less than 3% of both patients and controls were excluded from the association analysis. We performed 10,000 permutations for the most significant test to determine an empirical significance. We used a 2- to 5-window fashion analysis. Bonferroni corrections were applied for multiple comparisons of the haplotype analysis. All *P*-values reported are two tailed. Statistical significance was defined at *P* < 0.05.

### 2.4. Power analysis

We performed power calculations using the Power Calculator for Two Stage Association Studies (<http://www.sph.umich.edu/csg/abecasis/CaTS/>) (Skol et al., 2006). Power estimates were based on allele frequencies of the associated markers ranging from 0.15 (M19) to 0.38 (M20), the odds ratio ranging from 1.33 (M14) to 1.46 (M12) for the markers indicated by Chumakov et al. (2002) and an alpha level of 0.05. Power was calculated under prevalence of 0.01 using an additive or a multiplicative model, assuming various degrees of allele frequencies and the odds ratios of the markers.

## 3. Results

Our sample size of 1774 cases and 2092 controls had sufficient power (>0.99) to detect an effect of the odds ratio

**Table 2**  
Haplotype analysis of G72 between patients and controls.

LD <sup>a</sup>	SNP IDs <sup>b</sup>	Haplotypic global <i>P</i> value			
		Window size			
		2	3	4	5
block I	M12 (rs3916965)				
		0.93			
	M14 (rs3916967)		0.97		
		0.92		<b>0.03</b>	
	M15 (rs2391191)			<b>0.03</b>	0.05
block II	M19 (rs778294)		0.08	0.06	<b>0.03</b>
		<b>0.04</b>		0.06	
	M20 (rs3916970)		0.15		0.27
		0.23		0.47	
	M22 (rs778293)		0.28		0.25
		0.09		0.32	
	M23 (rs3918342)		0.06		
	0.10				
	M24 (rs1421292)				

LD, linkage disequilibrium.

<sup>a</sup>According to the result of LD analysis, we divided tightly linked SNPs into two LD blocks: block I (M12, M14 and M15), block II (M22, M23 and M24).

<sup>b</sup>The db SNP IDs equivalent to the M-SNP IDs designed by Chumakov et al. (2002) are shown in parentheses.

Haplotypes with frequencies < 3% in each group are excluded. Significant *P*-values (< 0.05) are in bold face.

(1.33 or more) described in the initial report for each SNP (Chumakov et al., 2002). Genotype and allele frequencies of eight SNPs located in the *G72* gene and the flanking regions are shown in Table 1. Genotyping completeness ranged from 98.5% (M15) to 99.5% (M12). No deviation from HWE was detected in cases and controls (data not shown). Significant differences in the genotype frequency of M22 ( $\chi^2=6.75$ ,  $P=0.034$ ) and in the allele frequencies of M22 ( $\chi^2=5.48$ ,  $P=0.019$ ), M23 ( $\chi^2=4.72$ ,  $P=0.030$ ) and M24 ( $\chi^2=4.35$ ,  $P=0.037$ ) between patients and controls were observed. However, the associations did not survive after correction for multiple testing (the effective number of independent marker loci: 6.0, M22 allelic association:  $P=0.11$  after SNPSpD correction). There was no allelic or genotypic association of the other five SNPs with schizophrenia.

Haplotype analysis showed associations in two-marker haplotypes: M15–M19 (global  $P=0.03$ ) and M19–M20 (global  $P=0.04$ ), three-marker haplotype: M14–M15–M19 (global  $P=0.03$ ), four-marker haplotype: M12–M14–M15–M19 (global  $P=0.03$ ), and five-marker haplotype: M14–M15–M19–M20–M22 (global  $P=0.03$ ) (Table 2). This weak evidence for association became negative after correction for multiple testing (22 independent global test, M15–M19 haplotypic association:  $P=0.66$  after Bonferroni correction).

M22 showed a strong LD with M24 and a moderate LD with M23 in controls, and similar LD results were obtained in patients (Supplementary Fig. 1). The LD pattern of our data was similar to that of other ethnic groups in the previous studies. The two strong LD structures (Block 1 and Block 2) observed in the present study were similar to those observed in previous studies (Detera-Wadleigh and McMahon, 2006; Li and He, 2007; Ma et al., 2006).

#### 4. Discussion

The purpose of the present study was to investigate the association between *G72* polymorphisms and schizophrenia in a large Japanese population, comparable to the sample size included in the meta-analysis of Li and He (2007). Eight *G72* SNPs, which have been associated with schizophrenia in previous studies, were examined in case-control subjects (Detera-Wadleigh and McMahon, 2006; Li and He, 2007; Shi et al., 2008). We failed to replicate the association of any *G72* polymorphism (M12, M14, M15, M19, M20, M22, M23 and M24) with schizophrenia after correction for multiple testing. Power analysis showed that our subjects had sufficient power ( $>0.99$ ) to detect an effect of the odds ratio (1.33 or more) for each SNP shown in the original study. The findings of the power calculation did not support the hypothesis that the eight SNPs in the *G72* gene are associated with schizophrenia in Japanese population.

Nominal associations of the alleles, M22, M23 and M24, and the genotype of M22 with schizophrenia in this study were no longer positive after correction for multiple testing. However, we discuss the direction of the association in the SNPs, as there are considerable discrepancies among studies. These three SNPs are located from 25.8 Kb to 54.8 Kb 3' downstream of the last exon (exon 5) of *G72*. These SNPs form a highly strong LD block whose pattern is similar among different ethnic groups. The first study by Chumakov et al. reported that the minor allele G of M22 was less frequent in

Canadian patients with schizophrenia (31%) than in controls (40%). However, the following studies have indicated the reverse direction of the association of M22 compared with the original study (patients vs. controls: Ma et al.; 47.5 vs. 39.1 in Scottish samples, 41.1 vs. 34.6 in Chinese samples). Our results indicated that the minor allele G of M22 was enriched in patients with schizophrenia (26.0) than in control (23.6). Consistent to the discrepancies among studies (different direction in European populations and same direction in Asian population), recent meta-analysis has reported significant evidence for the association of M22 with schizophrenia in Asian population, but not in European population (Ma et al., 2006; Shi et al., 2008). The direction of association of M23 has shown significant heterogeneity between individual studies in European populations (C allele at M23, patients vs. controls: Chumakov et al., 2002; 43 vs. 51 in Canadian samples, 40 vs. 49 in Russian sample, Korostishevsky et al., 2006; 37.7 vs. 56.7 in Ashkenazi Jewish samples, Schumacher et al., 2004; 53 vs. 46 in German samples, Ma et al., 2006; 59.3 vs. 46.4 in Scottish samples) (Shi et al., 2008). Our results (patients vs. controls: 43.6 vs. 41.2) were consistent with the direction of the association of M23 in an Asian population (Shi et al., 2008; 53.3 vs. 52.4), although the statistical significance was not evident. The direction of association of M24 among the previous studies was identical (patients vs. controls: Chumakov et al., 2002; 55 vs. 47 in Canadian samples, Schumacher et al., 2004; 56 vs. 50 in German samples). We firstly examined a possible association of M24 with schizophrenia in an Asian population and did not find association with schizophrenia (the frequency of the T allele at M24 in patients: 49.8 and controls: 47.4). This suggests that M24 is not likely to have ethnic heterogeneity between Asian and European populations.

Recently, a large scale genome-wide association study (GWAS) using 479 cases and 2937 controls reported that 12 SNPs (odds ratios ranging 1.27–2.06,  $P < 1 \times 10^{-5}$ ) were associated with schizophrenia in the first analysis (O'Donovan et al., 2008). The subsequent replication studies using 16,726 of total subjects showed the evidence for association with three of the 12 SNPs ( $P < 1 \times 10^{-5}$ ) (O'Donovan et al., 2008). They reported the odds ratios 1.12–1.16 in the three positive SNPs in the overall analysis (O'Donovan et al., 2008). The sample size in the first analysis of the GWAS had power ( $>0.85$ ) to detect an effect of the odds ratio (1.33 or more) for each SNP in the original *G72* study. However, association between SNPs in *G72* and schizophrenia was not detected in the study. Failure to detect association signal does not provide conclusive exclusion of any given gene identified so far. The discrepancies among previous studies and the present study might be explained by differences in ethnic heterogeneity, phenotypic heterogeneity or study designs, such as sample size and case-control versus family-based association study. Factors like incomplete coverage of common variants, inadequate power, allelic and locus heterogeneity could all affect our ability to detect genetic association.

In conclusion, the present study did not support a strong association of the *G72* gene with schizophrenia in a Japanese population. Three SNPs and several haplotypes gave nominal evidence for association and this did not survive correction for multiple testing. *G72* is not likely to be a major susceptibility gene for schizophrenia in this Japanese population.