Table 2
Allele frequencies and genotype of the Asn796Ser polymorphism of the BCR gene and response to lithium treatment

Response to lithium treatment	Allele frequency		χ^2test	Genotype		χ^2test
	Asn	Ser	p value (OR)	Asn/Asn Asn/Ser	Ser/Ser	p value
Responders (n=43)	49 (57.0%)	37 (43.0%)		35 (81.4%)	8 (18.6%)	
Non-responders (n=118)	101 (42.8%)	135 (57.2%)		77 (65.3%)	41 (34.7%)	
			0.024 (1.77)			0.049
Total patients (n=161)	150 (46.6%)	172 (53.4%)		112 (69.6%)	49 (30.4%)	

OR: Odds ratio.

2.2. Genotyping

Venous blood was drawn from the subjects and genomic DNA was extracted from whole blood according to the standard procedures. The genotype of the Asn796Ser SNP (rs140504) of the *BCR* gene was determined by TaqMan 5′-exonuclease allelic discrimination assay, described previously (Hashimoto et al., 2005). Briefly, probes and primers for detection of the polymorphism were: forward primer 5′-AGCTGGACGCTTTGAAGATCA-3′, reverse primer 5′-TGGTGTGCACCTTCTCTCTCT-3′, probe 1 5′-VIC-CCAGATCAAGAATGACAT-MGB-3′, and probe 2 5′-FAM-CCAGATCAAGAGTGACAT-MGB-3′. PCR cycling conditions were: at 95 °C for 10 min, 50 cycles of 92 °C for 15 s and 60 °C for 1 min.

2.3. Statistical analysis

Difference in clinical characteristics between responders and non-responders to lithium treatment was analyzed using the χ^2 tests for categorical variables and t tests for continuous variables. The presence of Hardy–Weinberg equilibrium was examined by using the χ^2 test for goodness of fit. Subsequently, multiple logistic regression analysis was performed to correct background difference between responders and non-responders for lithium treatment. Possible predictors (genotype of the *BCR* gene, subtype of bipolar disorder, age of onset, age at last observation, and gender) were included in the original model. Backward stepwise regression was performed, and p-value greater than 0.10 was used for variable removal. Pearson coefficient of correlation test was used for comparison between recurrence index and clinical variables. The effect of the Asn796Ser SNP on recurrence index was assessed by analysis of variance (ANOVA). All p-values reported are two-tailed. Statistical significance was defined at $p<0.05$.

3. Results

Among 161 patients with BPD, 43 patients were determined as responders and 118 patients as non-responders for the maintenance treatment of lithium. The clinical characteristics sorted by response to lithium treatment and genotype distribution were presented in Table 1. There were significant differences between responders and non-responders in subtype of bipolar disorder (BPI and BPII), age at last observation, and age of onset.

The genotype distributions for the total patients, responders, and non-responders were in Hardy–Weinberg equilibrium (total patients: $\chi^2=0.94$, $df=1$, $p=0.33$; responders: $\chi^2<0.001$, $df=1$, $p=0.98$; non-responders: $\chi^2=0.81$, $df=1$, $p=0.37$). Allele frequencies and genotype distributions of the Asn769Ser polymorphism of the *BCR* gene among responders and non-responders for lithium treatment are presented in Table 2. The Ser796 allele was in excess in the non-responders rather than responders ($\chi^2=5.09$, $df=1$, $p=0.024$; OR 1.77, 95% CI 1.08–2.92). Then, we examined patients homozygous for the Ser796 allele and the Asn796 allele carriers, separately. Patients homozygous for the Ser796 allele were significantly more common in the non-responders than the Asn796 carriers ($\chi^2=3.88$, $df=1$, $p=0.049$; OR 2.33, 95% CI 0.99–5.49). After backward stepwise regression, the final logistic regression model included subtype of bipolar ($p<0.01$), age of onset ($p<0.01$), and genotype which is separated to the Asn796 carrier and homozygous for the Ser796 ($P=0.04$).

We next investigated the association between lithium response using recurrence index and clinical variables in 24 subjects with BPD. The change of recurrence index before to during lithium treatment was not associated with subtype (t=0.79, $df=22$, $p=0.44$), age of onset (correlation coefficient=−0.29, $p=0.17$), duration from onset of illness to lithium treatment (correlation coefficient=0.12, $p=0.57$), duration during treatment (correlation coefficient=0.11, $p=0.60$), or the Asn796Ser SNP ($df=2$, $F=0.03$, $p=0.97$).

We also examined the association between age of onset and recurrence index before lithium treatment, which reflects severity of illness. There was a negative trend between age of onset and recurrence index (correlation coefficient=−0.37, $p=0.074$). Although difference among genotype of Asn796Ser SNP was not statistically significant, the number of Ser796 allele was associated with higher recurrence index before lithium treatment (Asn/Asn=1.63±1.19, Asn/Ser=2.89±0.84, and Ser/Ser=3.23±1.19. $df=2$, $F=0.53$, $p=0.60$). Therefore, the Ser796 allele might also be associated with both early onset and severity of illness, which could result in poorer lithium response.

4. Discussion

We investigated a possible association between the *BCR* gene and the prophylactic effect of lithium treatment in patients with BPD for the first time. As expected, our results suggested that lithium treatment might be less effective in patients homozygous for the Ser796 allele of the *BCR* gene than in patients with the Asn796 allele. In addition, allele frequencies of the Ser796 associated with poorer lithium response were 43.0%

in responders and 57.2% in non-responders. As allele frequency of the Ser796 in healthy subjects in our previous study was 48.1% (Hashimoto et al., 2005), allele frequency of the Ser796 of responders is similar to the general population.

Comparing clinical characteristics of responders and non-responders, there were more BPII patients in non-responder group. Clinical characteristics predicting poorer response to lithium therapy and that of BPII seem to overlap each other, but better lithium response in BPI is not universally accepted. We excluded any Axis I comorbidity in this study. This would leave in more typical bipolar II patients who would be more likely to respond to lithium, however, other clinical factors such as Axis II comorbidity might influence our results. The presence of positive family history of lithium responsive BPD has been reported as indicative of favorable response (Grof et al., 2002). However, it was not assumed that our sample size was enough to investigate this issue because only 8.7% of BPD had positive family history of the same disease in 1st degree relatives (Smoller and Finn, 2003). Therefore, information about family history of lithium response was not collected in this study.

Age at onset was also different between responders and non-responders, and early age of onset was associated with poorer response to lithium treatment in our subjects. This observation is consistent with recent meta-analysis (Kleindienst et al., 2005). As the objective of this study is to examine the association between response to lithium treatment and a SNP in the *BCR* gene, the differences in demographic parameters of responders and non-responders might not be preferable. Therefore, we conducted a multiple logistic regression analysis, and homozygous for the Ser796 allele of the *BCR* gene was still significantly associated with poorer response to lithium treatment.

The evaluation of lithium prophylaxis is considerably difficult because of complex clinical course of BPD, and each researcher has used different methodologies. Although our finding was based on the simple definition, in which lithium responders didn't have any affective recurrences during lithium, one of the limitation of this study is lack of detailed clinical information, e.g., duration from onset of illness to lithium treatment and number of episodes which could be clearly identified before lithium treatment in total subjects. To evaluate lithium efficacy including partial response, calculating recurrence index before and during lithium treatment is used in several researches. This would be a correct measure of lithium prophylaxis, but evaluating mood recurrence accurately before the first contact to mental professionals is difficult. We tried to evaluate lithium response with recurrence index; however, we could examine it in only 24 subjects out of 161 subjects due to the difficulty of collecting this clinical information. We did not found any association between the recurrence index and clinical variables and the SNP in the *BCR* gene, except for the trend between the recurrence index and age of onset. As these results were from subgroup analysis with smaller number, further investigation is needed in a larger sample size.

In this study, the same variant associated with the illness was also associated with poorer outcome. This situation is similar to that of the Val allele of the *BDNF* Val66Met polymorphism (Rybakowski et al., 2005), and it is possible that the *BCR* Ser796 and the *BDNF* Val66 alleles are associated with severer illness presentation. The trend between the recurrence index and age of onset in our subgroup analysis might imply this possibility. In case of the *BDNF* Val66Met SNP, the functional differences arisen from each allele were reported (Eagan et al., 2003). While biological functional of the *BCR* Asn796Ser SNP is still unknown, this SNP may produce functional difference in the brain, like the *BDNF* Val66Met SNP. To speculate this issue, it is noteworthy that this SNP is in the pleckstrin homology (PH) domain of the *BCR*. As PH domain is known for its ability to bind phosphatidylinositol and this binding regulates the activity of PH domain containing protein (Lemmon et al., 2002), signal transduction from inositol cycle to the *BCR* products might be affected by this SNP. As the *BCR* is RhoGAP, this change may influence on the activity of its downstream target, RhoGTPase, which activates many kind of effectors associated with constructing neuronal network, and subsequently influence on neuronal development. Additionally, as inositol cycle is considered as one of therapeutic targets of lithium (Harwood, 2005), this SNP could alter the clinical efficacy of lithium. To understand the mechanism of our findings, it is worth investigating whether the Asn796Ser SNP alters the binding ability of PH domain to inositol.

5. Conclusion

This is the first report demonstrating that long-term lithium treatment may be less effective in BPD patients homozygous for Ser796 allele of the *BCR* gene than in patients with the Asn796 allele. The limitations of this study are retrospective design without placebo control group, small sample size, and lack of clinical information such as presence of rapid cycling and/or psychotic symptoms, and detailed lithium levels. Further investigations are needed to confirm our findings.

Acknowledgements

The authors thank Ms. Tomoko Shizuno and Keiko Okada for their technical assistance. This work was supported in part by Grants-in-Aid from the Japanese Ministry of Health, Labor and Welfare, the Japanese Ministry of Education, Culture, Sports, Science and Technology, CREST (Core research for Evolutional Science and Technology) of JST (Japan Science and Technology Agency), Research on Health Sciences focusing on Drug Innovation of The Japan Health Sciences Foundation, and Japan Foundation for Neuroscience and Mental Health.

Duality of interest. The study sponsor had no involvement in study design, data collection, analysis, or interpretation, writing of the paper, or the decision to submit the paper for publication.

References

American Psychiatric Association. Practice guideline for the treatment of patients with bipolar disorder (revision). Am J Psychiatry 2002;159 (4 Suppl):1–50.

Ayuso-Gutierrez JL, Ramos-Brieva JA. The course of manic-depressive illness. A comparative study of bipolar I and bipolar II patients. J Affect Disord 1982;4:9–14.

Berk M, Dodd S. Bipolar II disorder: a review. Bipolar Disord 2005;7:11–21.

Del Zompo M, Ardau R, Palmas MA, Bocchetta A, Reina A, Piccardi MP. Lithium response: association study with two candidate genes. Mol Psychiatry 1999;4:S66–7.

Diekmann D, Brill S, Garrett MD, Totty N, Hsuan J, Monfries C, et al. Bcr encodes a GTPase-activating protein for p21rac. Nature 1991;351:400–2.

Eagan MF, Kojima M, Callicott JH, Goldberg TE, Kolachana BS, Bertolino A, et al. The BDNF val66met polymorphism affects activity-dependent secretion of BDNF and human memory and hippocampal function. Cell 2003;112:257–69.

Fioretos T, Voncken JW, Baram TZ, Kamme F, Groffen J, Heisterkamp N. Regional localization and developmental expression of the BCR gene in rodent brain. Cell Mol Biol Res 1995;41:97–102.

Gasperini M, Scherillo P, Manfredonia MG, Franchini L, Smeraldi E. A study of relapses in subjects with mood disorder on lithium treatment. Eur Neuropsychopharmacol 1993;3:103–10.

Gelenberg AJ, Pies R. Matching the bipolar patient and the mood stabilizer. Ann Clin Psychiatry 2003;15:203–16.

Grof P, Duffy A, Cavazzoni P, Grof E, Garnham J, MacDougall M, et al. Is response to prophylactic lithium a familial trait? J Clin Psychiatry 2002;63: 942–7.

Harwood AJ. Lithium and bipolar mood disorder: the inositol-depletion hypothesis revisited. Mol Psychiatry 2005;10:117–26.

Hashimoto R, Okada T, Kato T, Kosuga A, Tatsumi M, Kamijima K, et al. The breakpoint cluster region gene on chromosome 22q11 is associated with bipolar disorder. Biol Psychiatry 2005;57:1097–102.

Ikeda A, Kato T. Biological predictors of lithium response in bipolar disorder. Psychiatry Clin Neurosci 2003;57:243–50.

Kleindienst N, Engel R, Greil W. Which clinical factors predict response to prophylactic lithium? A systematic review for bipolar disorders. Bipolar Disord 2005;7:404–17.

Lemmon MA, Ferguson KM, Abrams CS. Pleckstrin homology domains and the cytoskeleton. FEBS Lett 2002;513:71–6.

Mamdani F, Sequeira A, Alda M, Grof P, Rouleau G, Turecki G. No association between the PREP gene and lithium responsive bipolar disorder. BMC Psychiatry 2007;26:7–9.

Masui T, Hashimoto R, Kusumi I, Suzuki K, Tanaka T, Nakagawa S, et al. Lithium response and Val66Met polymorphism of the brain-derived neurotrophic factor gene in Japanese patients with bipolar disorder. Psychiatr Genet 2006;16:49–50.

Michelon L, Meira-Lima I, Cordeiro Q, Miguita K, Breen G, Collier D, et al. Association study of the INPP1, 5HTT, BDNF, AP-2beta and GSK-3beta GENE variants and restrospectively scored response to lithium prophylaxis in bipolar disorder. Neurosci Lett 2006;403:288–93.

Negishi M, Katoh H. Rho family GTPases as key regulators for neuronal network formation. J Biochem (Tokyo) 2002;132:157–66.

Rybakowski JK, Suwalska A, Skibinska M, Szczepankiewicz A, Leszczynska-Rodziewicz A, Permoda A, et al. Prophylactic lithium response and polymorphism of the brain-derived neurotrophic factor gene. Pharmacopsychiatry 2005;38:166–70.

Serretti A, Lilli R, Mandelli L, Lorenzi C, Smeraldi E. Serotonin transporter gene associated with lithium prophylaxis in mood disorders. Pharmacogenomics J 2001;1:71–7.

Serretti A, Lorenzi C, Lilli R, Mandelli L, Pirovano A, Smeraldi E. Pharmacogenetics of lithium prophylaxis in mood disorders: analysis of COMT, MAO-A, and Gbeta3 variants. Am J Med Genet 2002;114:370–9.

Smoller JW, Finn CT. Family, twin, and adoption studies of bipolar disorder. Am J Med Genet C 2003;123:48–58.

Neuropsychopharmacology (2008), 1–9

www.neuropsychopharmacology.org

Genetic Variations of Human Neuropsin Gene and Psychiatric Disorders: Polymorphism Screening and Possible Association with Bipolar Disorder and Cognitive Functions

Aiko Izumi[1,2], Yoshimi Iijima[1], Hiroko Noguchi[1], Tadahiro Numakawa[1], Takeya Okada[1], Hiroaki Hori[1], Tadafumi Kato[3], Masahiko Tatsumi[4,5], Asako Kosuga[4], Kunitoshi Kamijima[4], Takashi Asada[6], Kunimasa Arima[7], Osamu Saitoh[7], Sadao Shiosaka[2] and Hiroshi Kunugi*[,1]

[1]Department of Mental Disorder Research, National Institute of Neuroscience, National Center of Neurology and Psychiatry, Tokyo, Japan; [2]Division of Structural Cell Biology, Nara Institute of Science and Technology, Nara, Japan; [3]Laboratory for Molecular Dynamics of Mental Disorders, Brain Science Institute, RIKEN, Saitama, Japan; [4]Department of Psychiatry, Showa University School of Medicine, Tokyo, Japan; [5]Yokohama Shinryo Clinic, Kanagawa, Japan; [6]Department of Psychiatry, Institute of Clinical Medicine, University of Tsukuba, Ibaraki, Japan; [7]Department of Psychiatry, Musashi Hospital, National Center of Neurology and Psychiatry, Tokyo, Japan

Human neuropsin (NP) (hNP) has been implicated in the progressive change of cognitive abilities during primate evolution. The hNP gene maps to chromosome 19q13, a region reportedly linked to schizophrenia and bipolar disorder. Therefore, hNP is a functional and positional candidate gene for association with schizophrenia, mood disorders, and cognitive ability. Polymorphism screening was performed for the entire hNP gene. The core promoter region was determined and whether or not transcriptional activity alters in an allele-dependent manner was examined by using the dual-luciferase system. Allelic and genotypic distributions of five single-nucleotide polymorphisms (SNPs) were compared between patients with schizophrenia ($n=439$), major depression ($n=409$), bipolar disorder ($n=207$), and controls ($n=727$). A possible association of the hNP genotype with memory index (assessed with Wechsler Memory Scale, revised, WMS-R) and intelligence quotient (IQ assessed with Wechsler Adult Intelligence Scale, revised; WAIS-R) was examined in healthy controls ($n=166$). A total of 28 SNPs, including nine novel SNPs, were identified. No significant effects on transcriptional activity were observed for SNPs in the promoter region. A significant allelic association was found between several SNPs and bipolar disorder (for SNP23 at the 3′ regulatory region; odds ratio 1.48, 95% confidential interval 1.16–1.88, $P=0.0015$). However, such an association was not detected for schizophrenia or depression. Significant differences were observed between SNP23 and attention/concentration sub-scale score of WMS-R ($P=0.016$) and verbal IQ ($P<0.001$). Genetic variation of the hNP gene may contribute to molecular mechanisms of bipolar disorder and some aspects of memory and intelligence.
Neuropsychopharmacology advance online publication, 19 March 2008; doi:10.1038/npp.2008.29

Keywords: neuropsin; polymorphism screening; association study; bipolar disorder; memory; intelligence

INTRODUCTION

Neuropsin (NP, MIM: 605644), also called as kallikrein 8 (KLK8), is one of the secreted-type serine proteases, which was first cloned by our group in mice (Chen *et al*, 1995). NP mRNA is expressed specifically in the limbic system of mouse brain and is localized at the highest concentration in pyramidal neurons of the hippocampal CA1-3 sub-fields. Direct hippocampal stimulation and kindling induced by amygdaloid stimulation caused a significant bilateral change in NP mRNA level in the hippocampal pyramidal neurons. The activity-dependent changes and the specific localization indicate that NP is involved in hippocampal plasticity (Chen *et al*, 1995). Indeed, NP has a regulatory effect on Schaffer-collateral at the early phase of long-term potentiation (LTP) (Komai *et al*, 2000). Mice lacking NP were significantly impaired in the Morris water maze and Y maze, suggesting that NP has an important role in learning and memory (Tamura *et al*, 2006). The human NP (hNP) gene was cloned by Yoshida *et al* (1998), and then localized to chromosome 19q13.3–q13.4 (Gan *et al*, 2000; Harvey *et al*, 2000). It consists of six exons and the first exon is non-translational. Four alternative splicing variants have been identified (Mitsui *et al*, 1999; Magklara *et al*, 2001). The regular form is called type1, and type 2 contains a 135-bp

*Correspondence: Dr H Kunugi, Department of Mental Disorder Research, National Institute of Neuroscience, National Center of Neurology and Psychiatry, 4-1-1, Ogawahigashi, Kodaira, Tokyo 187-8502, Japan, Tel/Fax: +81 42 346 1714, E-mail: hkunugi@ncnp.go.jp
Received 7 September 2007; revised 10 January 2008; accepted 7 February 2008

insertion of 5′ upstream region of exon 3 (Mitsui *et al*, 1999). Interestingly, type 2 is a hominoid-specific splicing form (Li *et al*, 2004) and is expressed as abundantly as the type 1 in human brain (Mitsui *et al*, 1999). These findings points to the possibility that type 2 hNP may contribute to progressive change of cognitive abilities during primate evolution. Moreover, dysfunctions in hNP may be involved in psychiatric diseases of cognitive abilities, including schizophrenia and mood disorders.

Family, twin, and adoption studies clearly suggest that genetic components play an important role in the pathogenesis of schizophrenia and mood disorders (reviewed by Shih *et al*, 2004). These psychiatric diseases demonstrate substantial cognitive deficits such as learning and memory (reviewed by Sharma and Antonova, 2003; Robinson *et al*, 2006; Green, 2006). A genome screen of linkage with bipolar disorder pedigrees provided evidence for susceptibility locus on chromosome 19q13 (Badenhop *et al*, 2002). Another genome scan in schizophrenia and bipolar pedigrees obtained an LOD ratio score of 1.5 at 19q13 in schizophrenic families (Macgregor *et al*, 2004). Therefore, the hNP gene is a good candidate gene for association with schizophrenia and mood disorders. Here we performed, for the first time, a polymorphism screening and association analysis of the hNP gene with schizophrenia, major depression, and bipolar disorder in a Japanese sample. A possible association of hNP with memory and intelligence in healthy subjects was also examined. In addition, we determined a core promoter region of the hNP gene and examined whether transcriptional activity varies in an allele-dependent manner.

MATERIALS AND METHODS

Subjects

Subjects for the association study were 439 patients with schizophrenia (240 males, mean age of 44.6 years (SD 14.0)), 409 patients with major depression (136 males, 53.3 years (15.9)), 207 patients with bipolar disorder (80 males, 50.2 years (14.7)), and 727 healthy controls (324 males, 43.5 years (16.4)). Among these, 104 patients with bipolar disorder and 108 controls were recruited around Shiga prefecture, approximately 350 km to the west of Tokyo, while the remaining 1570 subjects were recruited around Tokyo. Consensus diagnosis by at least two psychiatrists, one of whom was in charge of the patients, was made for each patient according to the Diagnostic and Statistical Manual of Mental Disorders, 4th edition (DSM-IV) criteria (American Psychiatric Association, 1994), on the basis of unstructured interviews and information from medical records. Control subjects were healthy volunteers who had no current or past contact to psychiatric services. Among them 213 controls were screened by the Japanese version of the Mini-International Neuropsychiatric Interview (Sheehan *et al*, 1998; Otsubo *et al*, 2005) by a research psychiatrist, whereas the remaining controls were not screened by such a structured interview. Participants were excluded if they had prior medical histories of central nervous system disease or severe head injury, or if they met the criteria for substance abuse or dependence, or mental retardation. All subjects were biologically unrelated Japanese. After description of the study, written informed consent was obtained from every subject. The study protocol was approved by institutional ethics committees.

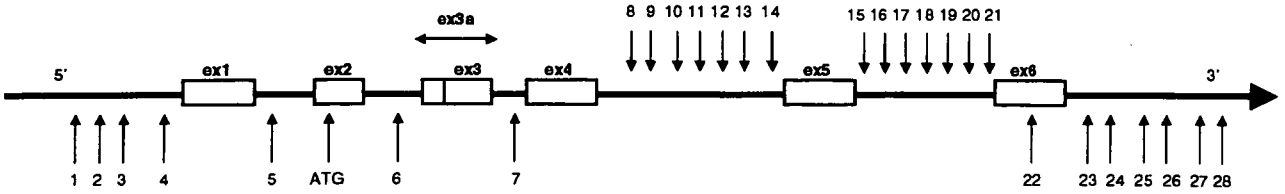
Neuropsychological Test Measures

Among controls, 166 (53 males, 37.6 years (12.4)) were subject to memory and intelligence tests to detect possible association with the hNP genotype. These individuals were all screened by the Mini-International Neuropsychiatric Interview with respect to their psychiatric history and confirmed that they had no current or past history of psychiatric illness. To assess memory and intelligence, Japanese full versions of the Wechsler Memory Scale-Revised (WMS-R) (Sugishita, 2001; Wechsler, 1987) and the Wechsler Adult Intelligence Scale-Revised (WAIS-R) (WAIS-R, Shinagawa *et al*, 1990; Wechsler, 1981), respectively, were administered. Testing and scoring were performed by psychologists who were blind to genotypic data.

Polymorphism Screening and Genotyping

Venous blood was drawn from the subjects and genomic DNA was extracted from whole blood according to standard procedures. The genomic structure of hNP was determined from the National Center for Biotechnology Information (NCBI) (http://www.ncbi.nlm.nih.gov/) and the University of California at Santa Cruz (UCSC) database (http://genome.ucsc.edu/cgi-bin/hgBlat). To screen for polymorphisms, we used direct sequencing with the Genome Lab-DTCS (Dye Terminator Cycle Sequencing) kit and CEQ8000 Genetic Analysis System (Beckman Coulter, Fullerton, CA). The entire 7428-bp genomic region containing all the exons, introns, the 1078-bp 5′ flanking region upstream to exon 1, and the 655-bp 3′ flanking region downstream to exon 6 were amplified from the genomic DNA of 24 randomly selected schizophrenic subjects. Sequences of 24 sets of primers for the polymorphism screening are listed in Supplementary Table S1.

The examined 7428-bp region seemed to constitute of single haplotype blocks (Supplementary Figure S1). We genotyped five single-nucleotide polymorphisms (SNPs) using TaqMan 5′-exonuclease allelic discrimination assay. They were A-807>G (ss73688625, SNP3), G-658>C (rs1722550, SNP4), IVS2-101T>A (rs1701947, SNP6), IVS3-10A>G (rs1701946, SNP7), and A5229>G (rs1612902, SNP23) (Figure 1). SNPs 3 and 4 were chosen from the 5′ regulatory region since they may have some effects on transcriptional activity, and SNPs 1, 2, 4, and 5 were in absolute linkage disequilibrium (LD) (ie, genotypes were completely the same) with each other. SNPs 6 and 7 were chosen because they were SNPs located close to the splicing sites of exon 3a (ie, an exon specific to type 2 hNP) and exon 4, respectively, and may have some effects on splicing. SNP23 was chosen from the 3′ region, since SNPs15, 16, 17, 19, 21, 23, 24, 25, 27, and 28 were in absolute LD with each other. TaqMan probes and Universal PCR master mix were obtained from Applied Biosystems (Foster City, CA). Thermal cycling conditions for polymerase chain reaction (PCR) were 1 cycle at 95°C for 10 min followed by 50 cycles of 92°C for 15 s and 60°C for 1 min. After



1. rs2569456	g.G-1302>T	15. rs1654539	g. IVS5+106G>A
2. rs6509509	g.C-948>T	16. rs1722559	g. IVS5+184C>T
3. ss73688625	g.A-807>G	17. rs1701914	g. IVS5+187T>C
4. rs1722550	g.G-658>C	18. rs1722560	g. IVS5+673T>G
5. ss73688633	g.IVS1+25,26 ins/del CA	19. rs1722561	g. IVS5+764T>C
6. rs1701947	g.IVS2-236T>A (exon3)	20. rs1701915	g. IVS5-442A>G
7. rs1701946	g.IVS3-10A>G	21. rs1627239	g. IVS5-355G>T
8. ss73688628	g.IVS4+596G>A	22. ss73688626	g. G5047>A (Val 286 Ile)
9. rs7245714	g.IVS4+866C>T	23. rs1612902	g. A5228>G
10. ss73688629	g.IVS4+1034C>T	24. rs1654542	g. T5308>C
11. ss73688630	g.IVS4-951A>G	25. rs1654543	g. A5435>G
12. rs12610313	g.IVS4-624C>A	26. ss73688627	g.A5579>G
13. ss73688631	g.IVS4-585C>T	27. rs1897605	g. T5646>C
14. ss73688632	g.IVS4-306T>C	28. rs1897606	g. C5777>T

Figure 1 Genomic structure and identified polymorphisms in the human NP gene. A total of 28 SNPs, including one insertion/deletion (ins/del) polymorphism, were identified. The A of the translational start ATG is designated + 1. Nine SNPs were novel and have been registered in the dbSNP (ss-tagged numbers). ex, exon; ex3a, exon3 in hNP type2.

amplification, the allele-specific fluorescence was measured with ABI PRISM 7900 Sequence Detection Systems (Applied Biosystems). Genotype data were read blind to the case-control status. Ambiguous genotype data were not included in the analysis.

Promoter Assay in Primary Cultured Neurons

Primary dissociated cultures were prepared from the brain cortex of postnatal 2-day-old rats (SLC, Shizuoka, Japan) as described previously (Numakawa *et al*, 2002). To generate plasmids for luciferase gene reporter assay, two differentially sized (964 and 128 bp) fragments of the 5′ flanking region of hNP were amplified by PCR with primers 5′-CGA CGCGTGCGTGTGCTGGGTTTGAA-3′ (forward) and 5′-GA AGATCTCTAGAGCCTGGGGAGCTTCT-3′ (reverse) for the 964-bp fragment, and 5′-CGACGCGTCCTCCTCTCCCTAGC CTCAG-3′ (forward) and 5′-GAAGATCTCTAGAGCCTGGG GAGCTTCT 3′ (reverse) for the 128-bp fragment. These primers were designed to incorporate *Mlu*I (forward) and *Bgl*II (reverse) restriction sites, and the PCR product was inserted into the multiple cloning site upstream of the luciferase coding region in the pGL3-Basic vector (Promega, Madison, WI). The inserted sequence was confirmed with the auto sequencer CEQ8000 in both directions using primers 5′-TCTCCATCAAAACAAAACGAA-3′and 5′-TTCC ATCTTCCAGCGGATA-3′.

Among the four SNPs (SNPs 1–4; see Figure 1) in the 5′ upstream region (ie, putative promoter region) of the hNP gene, the genotypes of SNPs 1, 2, and 4 were completely the same for all the 24 schizophrenic subjects, and we found a significant association of bipolar disorder with SNP4 but not SNP3 (see results). In addition, haplotypes containing the A allele (the major allele), but not the G-allele, of SNP3 showed some evidence for association with bipolar disorder in haplotype analysis (see Table 2). We therefore made two allele-specific promoter fragments (haplotypes consisting of SNPs 1-2-3-4 were G-C-A-G and T-T-A-C) of 964- and 128 bp upstream from the transcription initiation site, which were subject to the luciferase reporter gene assay. The plasmid constructs were transfected into cultured neurons at 5 days *in vitro*. Cells on 24-well plates were co-transfected with 3200 ng of pGL3-Basic firefly luciferase reporter vectors, which included allele-specific promoter fragments of 964 and 128 bp, and 100 ng of phRL-TK *Renilla* luciferase vector (Promega, Tokyo, Japan) as an internal control using Lipofectamine 2000 regent (Invitrogen, Tokyo, Japan). As negative control, an empty pGL3-Basic vector was simultaneously transfected in all the experiments. At 24 h after transfection, luciferase activity was measured using a Dual-Luciferase Reporter Assay System (Promega) and a Lumat LB 9507 luminometer (Berthold, Bad Wildbad, Germany), as described previously (Tadokoro *et al*, 2004; Okada *et al*, 2006). Firefly and *Renilla* luciferase activities were quantified sequentially as relative light unit (RLU) by addition of their respective substrates according to the protocol of the supplier. The ratio of firefly RLU to *Renilla* RLU of each sample was automatically computed. The activity of each construct was expressed at the relative value compared with that of pGL3-Promoter (as a positive control), and these relative values were computed by *t*-test. Primary cultured cells were prepared six times and transfection was performed quadruplicate for each cell culture.

Statistical Analysis

Deviations of genotype distributions from Hardy–Weinberg equilibrium were assessed with χ^2-test for goodness of fit. Genotype and allele distributions of each SNP were compared between patients and controls using χ^2-test for independence. The association of the hNP genotype with

memory and intelligence was examined by multiple analysis of variance (MANOVA) controlling for possible confounders (age, sex, and education years). These tests were performed with the SPSS software version 11 (SPSS Japan, Tokyo, Japan). The LD (D') between polymorphisms was examined using the Haploview program (http://www.broad.mit.edu/mpg/haploview/) (Barrett *et al*, 2005) and haplotype-based association analyses were performed with COCAPHASE software version 2.4 (http://www.mrc-bsu.cam.ac.uk/personal/frank/software/unphased/; Dudbridge *et al*, 2000). The expectation-maximization (EM) and 'droprare' options were used. Haplotypes with frequencies less than 3% were considered to be rare. We examined associations by permutation procedure (10 000 replications) to determine the empirical significance. All P-values reported are two-tailed. Statistical significance was considered when $P<0.05$.

RESULTS

Polymorphism Identification and Genotyping

The 7428-bp genomic region containing all the exons, introns, 5′ flanking, and 3′ flanking regions of hNP were screened for polymorphisms in 24 schizophrenic patients. A total of 28 SNPs, including one insertion/deletion (ins/del) polymorphism, were identified (Figure 1). Among them 19 SNPs had already been listed in the NCBI dbSNP database, whereas nine SNPs were novel. Four SNPs were located in 5′ upstream, one SNP in exon 6, six SNPs in 3′ downstream, and the remaining 17 polymorphisms in introns. There was only one SNP that resulted in an amino-acid change, SNP22 (G5047>A; Val286Ile: the number of the amino acid is according to NP_653088), which gave rise to a restriction site for *Acy*I and was located at an evolutionarily conserved (rodents through humans) residue. This non-synonymous polymorphism was found in only one schizophrenic patient. Additional genotyping was performed for 178 individuals with schizophrenia; however, there was no individual carrying the 286Ile allele, indicating that this amino-acid change is a rare mutation. The LD between SNPs is shown in Supplementary Figure S1, indicating that the entire genomic region consists of single haplotype block. Genotypes for SNPs 1, 2, 4, and 5, those for SNPs 8 and 9, those for SNPs 10 and 13, and those for SNPs15, 16, 17, 19, 21, 23, 24, 25, 27, and 28, respectively, were completely the same as each other for the 24 individuals.

Promoter Assay

We identified four SNPs in the 1078-bp 5′ upstream region of the hNP gene, and SNPs 1, 2, and 4 were found to be associated with bipolar disorder, memory, and intelligence quotient (IQ) (see below). Furthermore, to our knowledge, there is no information in the literature on the location of core promoter of the hNP gene. We therefore performed a promoter assay using the dual-luciferase system (Promega) in rat cultured cortical neurons and examined whether transcriptional activity alters in an allele-dependent manner. As shown in Figure 2a, pGL3-Basic vectors containing 128- and 964-bp fragments, which consisted of major alleles for the four SNPs, demonstrated substantially higher RLEs

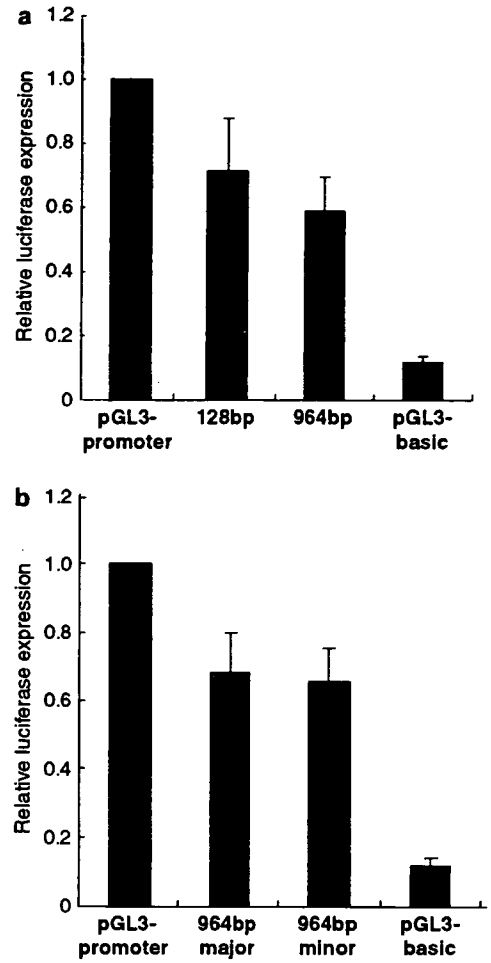


Figure 2 Promoter assay. (a) RLE for pGL3-Basic vector with insertion of 128 and 964 bp of the hNP 5′ flanking regions in comparison with pGL3-Basic vector, which dose not contain a promoter sequence. The RLE for pGL3-promoter vector containing SV40 promoter (positive control vector) was assigned a value of 1. Both 128- and 964-bp fragments showed substantially higher RLE compared with pGL3-basic vector without promoter sequence. (b) Comparison of RLE between the major (G-C-A-G for SNPs 1-2-3-4) and minor (T-T-A-C) alleles. No significant difference was found between the two alleles.

(relative luciferase expression) than that of pGL3-basic empty vector, suggesting that the core promoter region is located within the 128-bp fragment. We then cloned the 964-bp allele-specific promoter fragments (SNP1-2-3-4; major allele: G-C-A-G, minor allele: T-T-A-C) and compared RLEs between the two alleles (Figure 2b); however, we found no significant difference in the RLE between the two haplotype fragments. These results suggest that SNPs 1, 2, and 4 might not influence the transcriptional activity of the hNP gene.

Association with Psychiatric Diseases

We genotyped five SNPs (SNPs 3, 4, 6, 7, and 23) to examine possible association with schizophrenia, major depression, and bipolar disorder. Genotype and allele distributions in the diagnostic groups are shown in Table 1. Genotype

Table 1 Genotype and Allele Distributions of the five SNPs of the hNP Gene in Patients with Schizophrenia, those with Major Depression, those with Bipolar Disorder, and the Controls

SNP	Diagnosis	N	Genotype frequency (GF)			Allele frequency (AF)		Odds ratio (95% CI)	χ^2-Test vs controls		
									GF vs HW	GF (df = 2)	AF (df = 1)
SNP3			A/A	A/G	G/G	A	G				
	Controls	696	462 (0.66)	208 (0.30)	26 (0.04)	1132 (0.81)	260 (0.19)		0.67		
	SZ	421	277 (0.66)	126 (0.30)	18 (0.04)	680 (0.81)	162 (0.19)	1.06 (0.85–1.33)	0.45	$\chi^2 = 0.21$ $P = 0.90$	$\chi^2 = 0.11$ $P = 0.74$
	MD	382	276 (0.72)	90 (0.24)	16 (0.04)	642 (0.84)	122 (0.16)	1.18 (0.93–1.51)	0.02	$\chi^2 = 4.94$ $P = 0.08$	$\chi^2 = 2.48$ $P = 0.12$
	BD	202	139 (0.69)	56 (0.28)	7 (0.03)	334 (0.83)	70 (0.17)	1.09 (0.82–1.46)	0.65	$\chi^2 = 0.4$ $P = 0.81$	$\chi^2 = 0.36$ $P = 0.54$
SNP4			G/G	G/C	C/C	G	C				
	Controls	683	388 (0.57)	243 (0.36)	52 (0.08)	1019 (0.75)	347 (0.25)		0.11		
	SZ	406	234 (0.58)	150 (0.37)	22 (0.05)	618 (0.76)	194 (0.24)	1.1 (0.90–1.36)	0.75	$\chi^2 = 1.97$ $P = 0.37$	$\chi^2 = 0.62$ $P = 0.43$
	MD	371	219 (0.59)	126 (0.34)	26 (0.07)	564 (0.76)	178 (0.24)	1.1 (0.89–1.36)	0.19	$\chi^2 = 0.5$ $P = 0.78$	$\chi^2 = 0.58$ $P = 0.47$
	BD	198	91 (0.46)	90 (0.45)	17 (0.09)	272 (0.69)	124 (0.31)	1.33 (1.04–1.7)	0.43	$\chi^2 = 7.47$ $P = 0.023$	$\chi^2 = 5.35$ $P = 0.019$
SNP6			T/T	T/A	A/A	T	A				
	Controls	711	316 (0.44)	306 (0.43)	89 (0.13)	938 (0.66)	484 (0.34)		0.27		
	SZ	422	195 (0.46)	192 (0.45)	35 (0.08)	582 (0.69)	262 (0.31)	1.17 (0.97–1.41)	0.20	$\chi^2 = 4.86$ $P = 0.09$	$\chi^2 = 2.15$ $P = 0.14$
	MD	378	171 (0.45)	164 (0.43)	43 (0.11)	506 (0.67)	250 (0.33)	1.06 (0.88–1.29)	0.70	$\chi^2 = 0.31$ $P = 0.86$	$\chi^2 = 0.21$ $P = 0.65$
	BD	197	70 (0.36)	99 (0.50)	28 (0.14)	239 (0.61)	155 (0.39)	1.25 (0.99–1.58)	0.46	$\chi^2 = 5.03$ $P = 0.08$	$\chi^2 = 3.8$ $P = 0.051$
SNP7			A/A	A/G	G/G	A	G				
	Controls	718	325 (0.45)	314 (0.44)	79 (0.11)	964 (0.67)	472 (0.33)		0.81		
	SZ	433	209 (0.48)	190 (0.44)	34 (0.08)	608 (0.70)	258 (0.30)	1.16 (0.96–1.40)	0.31	$\chi^2 = 3.26$ $P = 0.20$	$\chi^2 = 2.36$ $P = 0.12$
	MD	387	182 (0.47)	163 (0.42)	42 (0.11)	527 (0.68)	247 (0.32)	1.05 (0.87–1.28)	0.55	$\chi^2 = 0.33$ $P = 0.85$	$\chi^2 = 0.21$ $P = 0.65$
	BD	203	72 (0.35)	103 (0.51)	28 (0.14)	247 (0.61)	159 (0.39)	1.31 (1.04–1.65)	0.36	$\chi^2 = 6.3$ $P = 0.042$	$\chi^2 = 5.56$ $P = 0.018$
SNP23			A/A	A/G	G/G	A	G				
	Controls	714	428 (0.60)	241 (0.34)	45 (0.06)	1097 (0.77)	331 (0.23)		0.16		
	SZ	421	267 (0.63)	135 (0.32)	19 (0.05)	669 (0.79)	173 (0.21)	1.17 (0.94–1.44)	0.71	$\chi^2 = 2.25$ $P = 0.32$	$\chi^2 = 2.13$ $P = 0.14$
	MD	388	240 (0.62)	127 (0.33)	21 (0.05)	607 (0.78)	169 (0.22)	1.08 (0.87–1.34)	0.44	$\chi^2 = 0.56$ $P = 0.75$	$\chi^2 = 0.56$ $P = 0.45$
	BD	204	98 (0.48)	86 (0.42)	20 (0.10)	282 (0.69)	126 (0.31)	1.48 (1.16–1.88)	0.86	$\chi^2 = 9.82$ $P = 0.0073$	$\chi^2 = 10.07$ $P = 0.0015$

Abbreviations: 95% CI, 95% confidence interval; BD, bipolar disorder; df, degrees of freedom; hNP, human neuropsin; HW, Hardy–Weinberg; MD, major depression; SNP, single-nucleotide polymorphism; SZ, schizophrenia.Significant *p*-values are gray colored.

distributions of these SNPs did not deviate significantly from Hardy–Weinberg equilibrium, except for SNP3 in patients with major depression ($P = 0.02$). There was no significant difference in genotype or allele distribution for any SNP between patients and controls for schizophrenia or major depression. However, there was a significant difference in genotype distributions between patients with bipolar disorder and controls for three SNPs, that is, SNPs 4, 7, and 23. Allele frequencies for these SNPs also differed significantly between the two groups. *P*-values, odds ratios, and their 95% confidence interval (CI) are shown in Table 1. Then we performed haplotype-based analysis with a two-marker sliding window method. We obtained no evidence of a significant association for schizophrenia or major depression (data not shown). With respect to bipolar disorder, we obtained significant individual *P*-values for all combinations of two markers; however, significant global *P*-value (0.0068) was obtained only when haplotype consisted of SNPs 7 and 23 (Table 2). Furthermore, overall global *P*-value ($P = 0.083$), considering all multiple testing for all the combinations of two-marker haplotypes, just failed to reach statistical significance. Thus, we did not obtain any stronger evidence for association in the haplotype-based analysis than in the single-marker analysis of SNP23 ($P = 0.0015$).

Association with Memory and IQ

Among the 166 controls whose memory scale and IQ were measured, SNP23 (A/G) was successfully genotyped in 163 individuals. Mean (SD) index scores of verbal memory, visual memory, general memory, attention and concentration, and delayed recall in the 163 controls were 110.9 (13.7), 109.9 (9.0), 112.2 (12.1), 103.9 (13.5), and 112.1

Table 2 Two-Marker Haplotype Analysis in Patients with Bipolar Disorder and Controls.

Markers					Haplotype frequency			P-value	
SNP3	SNP4	SNP6	SNP7	SNP23	BD	Controls	Individual	Global[a]	Overall global[a]
A	C				0.31	0.26	0.028	0.073	
	C	A			0.30	0.24	0.030	0.10	
		A	G		0.39	0.33	0.028	0.11	
			G	G	0.30	0.23	0.0068	0.014	0.083

Abbreviations: BD, bipolar disorder; SNP, single-nucleotide polymorphism.
[a]Global *P*-value for each combination of two markers and overall global significance for all combinations of two markers were calculated by permutation of 10000 simulations.

(12.0), respectively. Mean (SD) full-scale IQ, verbal IQ, and performance IQ were 109.3 (11.6), 107.3 (12.9), and 110.3 (11.7), respectively. Since SNP23 showed the strongest association with bipolar disorder (G-allele was the risk allele) among the 5 SNPs examined, memory and IQ were compared between those who carried the G-allele (carrier, G/G or A/G, $N=64$) and those who did not (non-carrier, A/A, $N=99$) (Figure 3). Since the number of individuals with G/G genotype was very small ($N=8$), they were combined with those with the A/G genotype. With respect to sub-scales of WMS-R, the mean score of attention/concentration was significantly lower in carriers than in non-carriers ($P=0.016$); however, there were no significant differences between the two groups for the remaining sub-scales (verbal memory, visual memory, general memory, and delayed recall). With respect to WAIS-R, there was a significant difference in full-scale IQ ($P=0.018$) between the two groups. When verbal and performance IQ were examined separately, there was a highly significant difference in verbal IQ ($P<0.001$), but not in performance IQ, between the two groups. The mean verbal IQ (SD) for carriers and non-carriers was 103.4 (12.9) and 109.8 (12.3), respectively. As for the other SNPs, similar results are obtained (data not shown) because of the tight LD across the SNPs.

DISCUSSION

In the present study, we performed polymorphism screening and identified 28 SNPs, including nine novel SNPs, in the 7428-bp region of the whole hNP gene, including the 5′ and 3′ flanking regions. Then we performed promoter assay and determined a core promoter region of the hNP gene, although failing to find significant effects of SNPs on transcriptional activity. Association analysis using five SNPs as markers revealed significant difference in genotype and allele distributions for some of the SNPs between patients and controls for bipolar disorder, but not for schizophrenia or major depression. When a possible association of the SNPs with memory and IQ was examined in healthy control subjects, we found significant differences in attention/concentration sub-scale score of the WMS-R and verbal IQ between genotypes.

Among the 28 SNPs identified, there was only one SNP in the exons; SNP22 was a Val286Ile missense mutation in exon 6, which was detected in a patient with schizophrenia. Additional genotyping for 178 schizophrenic subjects did not find anyone carrying this variant, indicating that this is a rare mutation. Thus, whether this mutation is pathogenic or not is unclear. Since we examined only 24 individuals for polymorphism screening, we may have missed some rare mutations as the SNP22.

Our promoter assay in rat primary cultured neurons suggested that the core promoter is present in the 128-bp 5′ upstream region of the hNP gene. Since the RLE of pGL3-vector containing the 964-bp fragment was somewhat lower than that of pGL3-vector containing 128-bp fragment, a silencer-like region may be present between 128- and 964-bp positions upstream of the hNP gene. Then we examined whether transcriptional activity differs in an allele-dependent manner; however, we found no significant difference between alleles. These results suggest that SNPs 1, 2, and 4 might not influence the transcriptional activity of the hNP gene. According to the TFSEARCH database (http://mbs.cbrc.jp/research/db/TFSEARCHJ.html), these SNPs are not located on any of the binding sites of transcriptional factors, which is in line with our finding of no significant difference between the alleles.

In our association study with psychiatric diseases, we found, for the first time, significant differences in genotype and allele distributions between patients with bipolar disorder and controls. The best *P*-value was obtained for SNP23 in allele distribution ($P=0.0015$, odds ratio 1.48, 95% CI 1.16–1.88). This *P*-value remained significant even after correcting the critical *P*-value for Bonferroni's multiple testing (15 comparisons: 5 SNPs × 3 diseases). Haplotype-based analysis also yielded nominally significant results particularly when SNP23 was included in markers of analysis. These results suggest that SNP23 or other unknown SNPs in LD with SNP23 confers susceptibility to bipolar disorder. Since hNP is a part of a gene cluster (kallikreins), there remains a possibility that variations of some other kallikrein gene might be truly responsible to giving susceptibility to bipolar disorder. The results are in line with a previous study reporting a susceptibility locus for bipolar disorder on chromosome 19q13 (Badenhop *et al*, 2002). A possible limitation is that a portion of patients with bipolar disorder and controls were recruited in a geographically different area (ie, Shiga prefecture but not in Tokyo), which may have resulted in a population stratification; however, the minor allele frequency of SNP23 was very similar in controls from Shiga and those from Tokyo (0.233 in Shiga and 0.232 in Tokyo), suggesting that the effect of stratification is unlikely. Another limitation might be that

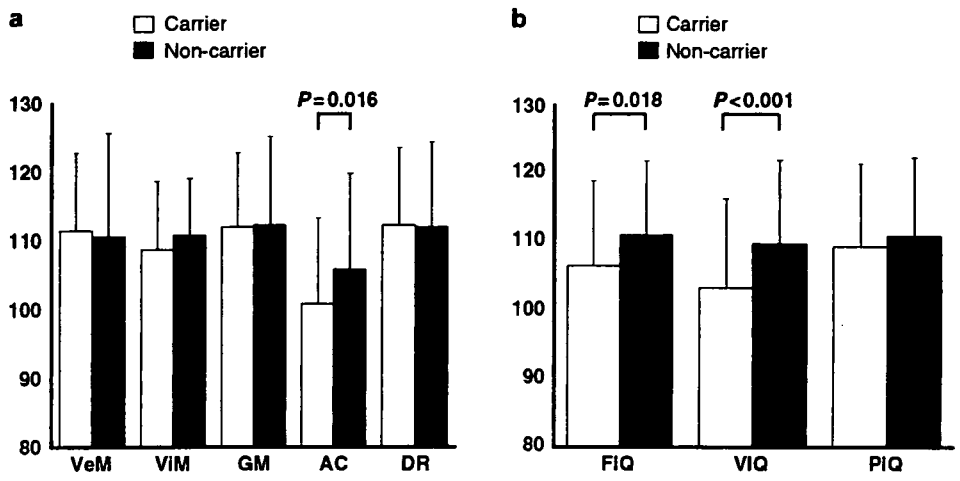


Figure 3 Relationship of memory and IQ with genotype of SNP23. Memory and IQ were compared between those who carried the G allele (carrier: G/G or A/G) of SNP23 and those who did not (non-carrier: A/A). (a) Memory and genotype. VeM, verbal memory; ViM, visual memory; GM, general memory; AC, attention and concentration; DR, delayed recall. (b) IQ and genotype. FIQ, full-scale IQ; VIQ, verbal IQ; PIQ, performance IQ.

we did not conduct structured interview for diagnosis of the patients. However, consensus diagnosis was made by at least two psychiatrists one of whom was in charge of the patients; thus, the possibility of misdiagnosis might be minimal. In addition, our sample size (207 bipolar disorder subjects and 727 controls) was not very large, and thus further investigations in other samples are required to draw any conclusion. With respect to schizophrenia or major depression, we did not obtain any evidence for association with hNP.

Interestingly, we found significant association of memory and IQ with the hNP gene in healthy subjects. Carrying the G-allele of SNP23, the risk allele for bipolar disorder, was associated with lower score in attention/concentration assessed with the WMS-R ($P=0.016$) and lower verbal IQ assessed with WAIS-R ($P<0.001$). The evidence for the former association (with attention/concentration) was weak and it would not be significant any more after correcting for multiple testing; however, the latter association (with verbal IQ) was highly significant and remained significant even when multiple testing was taken into consideration. Since bipolar disorder shows a wide range of cognitive deficits, including memory and IQ (Schretlen *et al*, 2007; Daban *et al*, 2006), the observed impact on intelligence may have some relevance to susceptibility to bipolar disorder. However, given that deficits in intelligence and memory are generally worse in schizophrenia than in bipolar disorder, alterations in hNP may have some effects specific to molecular mechanisms of bipolar disorder.

NP is a secretory serine protease that degrades cell adhesion molecule L1 (CAM-L1) (Matsumoto-Miyai *et al*, 2003) and is possibly involved in the synaptogenesis and maturation of orphan and small synapses (Nakamura *et al*, 2006). Furthermore, NP has been shown to be involved in activity-dependent synaptic plasticity, that is, LTP and kindling epileptogenesis (Komai *et al*, 2000; Okabe *et al*, 1996). As mentioned above, the type 2 splice variant has been shown to be expressed as abundant as the type 1 in human brain (Mitsui *et al*, 1999) and the hominoid-specific form (Li *et al*, 2004), which occurred through a human-specific T-to-A mutation (c.71-127T>A) during primate evolution (Lu *et al*, 2007). Taken together, NP is involved in synaptic plasticity via modulation of synaptic structure, and may play an important role in brain function of higher order such as learning, memory, and mental disorders. With respect to psychiatric diseases, indeed, altered expression levels of CAM-L1 mRNA and protein have been reported in postmortem brains of depressed patients (Laifenfeld *et al*, 2005). In line with this, chronic antidepressants increase expression levels of CAM-LI in rats (Sairanen *et al*, 2007; Laifenfeld *et al*, 2002). It would be intriguing to examine the expression levels of NP in postmortem brains of psychiatric patients.

We found that SNP23 is most associated with bipolar disorder among the examined SNPs. Haplotype-based analysis did not yield any stronger results, suggesting that SNP23 may be responsible for giving susceptibility to bipolar disorder. In addition, SNP23 showed strong impact on verbal IQ in healthy subjects. SNP23 is located 69 bp downstream to the 3′ end of exon 6 (the final exon). Thus SNP23 is on the 3′ regulatory region of the hNP gene. Growing evidence has shown that 3′ regulatory regions of human genes play an important role in regulating mRNA 3′ end formation, stability/degradation, nuclear export, subcellular localization, and translation, and are consequently rich in regulatory elements. Indeed, several diseases have been reported to be associated with variants in the 3′ regulatory region (Chen *et al*, 2006). Notably, the major allele (A) of SNP23 differs from the corresponding base (G) in monkeys or apes (ie, rhesus macaques or chimpanzees), according to the UCSC database, and thus it is not evolutionally conserved. It is interesting that carriers of the G allele were found to be poorer in memory and IQ subscales than individuals with A/A genotype in the present study. Although SNP23 is not located on obvious motifs or conserved sequence elements, it is also possible that this human-specific mutation may contribute to the higher memory and intelligence functions in humans. If our results

are replicated in other samples, it is important to elucidate the possible functional effects of SNP23 on regulation of hNP mRNA, which may contribute to understanding of the pathogenesis of bipolar disorder and brain function of higher order specific to human beings.

ACKNOWLEDGEMENTS

This study was supported by Health and Labor Sciences Research Grants (Research on Psychiatric and Neurological Diseases and Mental Health; Research on Human Genome and Tailor-made medicine), the Program for Promotion of Fundamental Studies in Health Sciences of the National Institute of Biomedical Innovation (NIBIO), Japan Health Sciences Foundation (Research on Health Sciences focusing on Drug innovation), Grant-in-Aid for Scientific Research on Priority Areas of Applied Genomics from the Ministry of Education, Culture, Sports, Science and Technology of Japan. We thank Ms Tomoko Shizuno for technical assistance.

DISCLOSURE/CONFLICT OF INTEREST

All authors declare that they have no conflict of interests to disclose.

REFERENCES

American Psychiatric Association (1994). *Diagnostic and Statistical Manual of Mental Disorders*, 4th edn. American Psychiatric Press: Washington, DC.

Badenhop RF, Moses MJ, Scimone A, Mitchell PB, Ewen-White KR, Rosso A *et al* (2002). A genome screen of 13 bipolar affective disorder pedigrees provides evidence for susceptibility loci on chromosome 3 as well as chromosomes 9, 13 and 19. *Mol Psychiatry* 7: 851–859.

Barrett JC, Fry B, Maller J, Daly MJ (2005). Haploview: analysis and visualization of LD and haplotype maps. *Bioinformatics* 21: 263–265.

Chen JM, Férec C, Cooper DN (2006). A systematic analysis of disease-associated variants in the 3′ regulatory regions of human protein-coding genes I: general principles and overview. *Hum Genet* 120: 1–21.

Chen ZL, Yoshida S, Kato K, Momota Y, Suzuki J, Tanaka T *et al* (1995). Expression and activity-dependent changes of a novel limbic-serine protease gene in the hippocampus. *J Neurosci* 15: 5088–5097.

Daban C, Martinez-Aran A, Torrent C, Tabarés-Seisdedos R, Balanzá-Martínez V, Salazar-Fraile J *et al* (2006). Specificity of cognitive deficits in bipolar disorder *versus* schizophrenia. A systematic review. *Psychother Psychosom* 75: 72–84.

Dudbridge F, Koeleman BP, Todd JA, Clayton DG (2000). Unbiased application of the transmission/disequilibrium test to multilocus haplotypes. *Am J Hum Genet* 66: 2009–2012.

Gan L, Lee I, Smith R, Argonza-Barrett R, Lei H, McCuaig J *et al* (2000). Sequencing and expression analysis of the serine protease gene cluster located in chromosome 19q13 region. *Gene* 257: 119–130.

Green MF (2006). Cognitive impairment and functional outcome in schizophrenia and bipolar disorder. *J Clin Psychiatry* 67: 3–8.

Harvey TJ, Hooper JD, Myers SA, Stephenson SA, Ashworth LK, Clements JA (2000). Tissue-specific expression patterns and fine mapping of the human kallikrein (KLK) locus on proximal 19q13.4. *J Biol Chem* 275: 37397–37406.

Komai S, Matsuyama T, Matsumoto K, Kato K, Kobayashi M, Imamura K *et al* (2000). Neuropsin regulates an early phase of Schaffer-collateral long-term potentiation in the murine hippocampus. *Eur J Neurosci* 12: 1479–1486.

Laifenfeld D, Karry R, Klein E, Ben-Shachar D (2005). Alterations in cell adhesion molecule L1 and functionally related genes in major depression: a postmortem study. *Biol Psychiatry* 57: 716–725.

Laifenfeld D, Klein E, Ben-Shachar D (2002). Norepinephrine alters the expression of genes involved in neuronal sprouting and differentiation: relevance for major depression and antidepressant mechanisms. *J Neurochem* 83: 1054–1064.

Li Y, Qian YP, Yu XJ, Wang YQ, Dong DG, Sun W *et al* (2004). Recent origin of a hominoid-specific splice form of neuropsin, a gene involved in learning and memory. *Mol Biol Evol* 21: 2111–2115.

Lu ZX, Peng J, Su B (2007). A human-specific mutation leads to the origin of a novel splice form of neuropsin (KLK8), a gene involved in learning and memory. *Hum Mutat* 28: 978–984.

Macgregor S, Visscher PM, Knott SA, Thomson P, Porteous DJ, Millar JK *et al* (2004). A genome scan and follow-up study identify a bipolar disorder susceptibility locus on chromosome 1q42. *Mol Psychiatry* 9: 1083–1090.

Magklara A, Scorilas A, Katsaros D, Massobrio M, Yousef GM, Fracchioli S *et al* (2001). The human KLK8 (neuropsin/ovasin) gene: identification of two novel splice variants and its prognostic value in ovarian cancer. *Clin Cancer Res* 7: 806–811.

Matsumoto-Miyai K, Ninomiya A, Yamasaki H, Tamura H, Nakamura Y, Shiosaka S (2003). NMDA-dependent proteolysis of presynaptic adhesion molecule L1 in the hippocampus by neuropsin. *J Neurosci* 23: 7727–7736.

Mitsui S, Tsuruoka N, Yamashiro K, Nakazato H, Yamaguchi N (1999). A novel form of human neuropsin, a brain-related serine protease, is generated by alternative splicing and is expressed preferentially in human adult brain. *Eur J Biochem* 260: 627–634.

Nakamura Y, Tamura H, Horinouchi K, Shiosaka S (2006). Role of neuropsin in formation and maturation of Schaffer-collateral L1cam-immunoreactive synaptic boutons. *J Cell Sci* 119: 1341–1349.

Numakawa T, Yamagishi S, Adachi N, Matsumoto T, Yokomaku D, Yamada M *et al* (2002). Brain-derived neurotrophic factor-induced potentiation of Ca(2+) oscillations in developing cortical neurons. *J Biol Chem* 277: 6520–6529.

Okabe A, Momota Y, Yoshida S, Hirata A, Ito J, Nishino H *et al* (1996). Kindling induces neuropsin mRNA in the mouse brain. *Brain Res* 728: 116–120.

Okada T, Hashimoto R, Numakawa T, Iijima Y, Kosuga A, Tatsumi M *et al* (2006). A complex polymorphic region in the brain-derived neurotrophic factor (BDNF) gene confers susceptibility to bipolar disorder and affects transcriptional activity. *Mol Psychiatry* 11: 695–703.

Otsubo T, Tanaka K, Koda R, Shinoda J, Sano N, Tanaka S *et al* (2005). Reliability and validity of Japanese version of the Mini-International Neuropsychiatric Interview. *Psychiatry Clin Neurosci* 59: 517–526.

Robinson LJ, Thompson JM, Gallagher P, Goswami U, Young AH, Ferrier IN *et al* (2006). A meta-analysis of cognitive deficits in euthymic patients with bipolar disorder. *J Affect Disord* 93: 105–115.

Sairanen M, O'Leary OF, Knuuttila JE, Castrén E (2007). Chronic antidepressant treatment selectively increases expression of plasticity-related proteins in the hippocampus and medial prefrontal cortex of the rat. *Neuroscience* 144: 368–374.

Schretlen DJ, Cascella NG, Meyer SM, Kingery LR, Testa SM, Munro CA *et al* (2007). Neuropsychological functioning in bipolar disorder and schizophrenia. *Biol Psychiatry* 62: 179–186.

Sharma T, Antonova L (2003). Cognitive function in schizophrenia. Deficits, functional consequences, and future treatment. *Psychiatr Clin North Am* **26**: 25–40.

Sheehan DV, Lecrubier Y, Sheehan KH, Amorim P, Janavs J, Weiller E *et al* (1998). The mini-international neuropsychiatric interview (M.I.N.I.): the development and validation of a structured diagnostic psychiatric interview for DSM-IV and ICD-10. *J Clin Psychiatry* **59**(Suppl 20): 22–57.

Shih RA, Belmonte PL, Zandi PP (2004). A review of the evidence from family, twin and adoption studies for a genetic contribution to adult psychiatric disorders. *Int Rev Psychiatry* **16**: 260–283.

Shinagawa F, Kobayashi S, Fujita K, Maekawa H (1990). *Japanese Wechsler Adult Intelligence Scale-Revised.* Nihonbunkakagakusha: Tokyo. (in Japanese).

Sugishita M (2001). *Japanese Wechsler Memory Scale-Revised.* Nihonbunkakagakusha: Tokyo. (in Japanese).

Tadokoro K, Hashimoto R, Tatsumi M, Kamijima K, Kunugi H (2004). Analysis of enhancer activity of a dinucleotide repeat polymorphism in the neurotrophin-3 gene and its association with bipolar disorder. *Neuropsychobiology* **50**: 206–210.

Tamura H, Ishikawa Y, Hino N, Maeda M, Yoshida S, Kaku S *et al* (2006). Neuropsin is essential for early processes of memory acquisition and Schaffer collateral long-term potentiation in adult mouse hippocampus *in vivo*. *J Physiol* **570**: 541–551.

Wechsler D (1981). *Wechsler Adult Intelligence Scale, Revised.* Psychological Corporation: New York.

Wechsler D (1987). *Wechsler Memory Scale Manual, Revised.* Psychological Corporation: San Antonio.

Yoshida S, Taniguchi M, Hirata A, Shiosaka S (1998). Sequence analysis and expression of human neuropsin cDNA and gene. *Gene* **213**: 9–16.

Supplementary Information accompanies the paper on the Neuropsychopharmacology website (http://www.nature.com/npp)

ARTICLE IN PRESS

Available online at www.sciencedirect.com
ScienceDirect
Journal of Psychiatric Research xxx (2007) xxx–xxx
JOURNAL OF PSYCHIATRIC RESEARCH
www.elsevier.com/locate/jpsychires

TGFBR2 gene expression and genetic association with schizophrenia

Shusuke Numata [a,*], Shu-ichi Ueno [a,b], Jun-ichi Iga [a], Ken Yamauchi [a], Song Hongwei [a], Ryota Hashimoto [c,d,e], Masatoshi Takeda [c,d], Hiroshi Kunugi [e], Mitsuo Itakura [f], Tetsuro Ohmori [a]

[a] *Department of Psychiatry, Course of Integrated Brain Sciences, Medical Informatics, Institute of Health Biosciences, The University of Tokushima Graduate School, 3-8-15 Kuramoto-cho Tokushima 770-8503, Japan*
[b] *Department of Community and Psychiatric Nursing, Major in Nursing, School of Health Sciences, The University of Tokushima Graduate School, 3-8-15 Kuramoto-cho Tokushima 770-8503, Japan*
[c] *The Osaka-Hamamatsu Joint Research Center For Child Mental Development, Osaka University Graduate school of Medicine, Japan*
[d] *Department of Psychiatry, Osaka University Graduate school of Medicine, Japan*
[e] *Department of Mental Disorder Research, National Institute of Neuroscience, National Center of Neurology and Psychiatry, Tokyo, Japan*
[f] *Division of Genetic Information, Institute for Genome Research, The University of Tokushima Graduate School, 3-8-15 Kuramoto-cho Tokushima 770-8503, Japan*

Received 12 October 2006; received in revised form 7 March 2007; accepted 18 April 2007

Abstract

TGFBR2 gene is a tumor suppressor gene located at chromosome 3p22, and the locus is reported to be linked with schizophrenia susceptibility. According to the previous studies, a reduced incidence of cancer is observed in schizophrenic patients compared with the general population and tumor suppressor genes may be associated with schizophrenia. We measured the mRNA expression of TGFBR2 gene in the peripheral leukocytes from 19 medication-free schizophrenics and 25 medication-free major depressive patients compared with age- and sex-matched control subjects using a quantitative real-time PCR method. We also followed up the TGFBR2 mRNA expression levels from 13 schizophrenics after several weeks – antipsychotic treatments. The TGFBR2 mRNA levels of medication free schizophrenics were significantly higher than those of control subjects and decreased to almost the same level as controls after antipsychotic treatment. On the other hand, the TGFBR2 mRNA levels of medication-free major depressive patients were not significantly different from controls. In genetic studies, we failed to find any association between the TGFBR2 gene and schizophrenia with 10 SNPs of TGFBR2 gene in Japanese subjects (279 subjects each) and there was no significant difference with haplotype analysis, either. Our results suggest that the TGFBR2 gene itself does not link to schizophrenia but that the TGFBR2 mRNA levels in the peripheral leukocytes may be a potential state marker for schizophrenia.

Keywords: TGFBR2; Gene expression; Leukocytes; Association analysis; Schizophrenia

1. Introduction

Schizophrenia is a complex psychiatric disorder that afflicts approximately 1% of the population throughout the world and has high heritability (Craddock et al., 2005). According to the previous studies, a reduced incidence of cancer is observed in schizophrenic patients compared with the general population (Catts and Catts, 2000; Grinshpoon et al., 2005). The possibility is explored to understand that alteration of the expression of oncogenes and/or tumor suppressor genes may account for tumor resistance associated with schizophrenia. Cui et al. reported that the tumor suppressor adenomatous polyposis coli (APC), which is involved in cell adhesion, was associated with schizophrenia and its expression levels were significantly increased in the leukocytes of schizo-

* Corresponding author. Tel.: +81 886 33 7130; fax: +81 886 33 7131.
E-mail address: snumata@clin.med.tokushima-u.ac.jp (S. Numata).

0022-3956/$ - see front matter © 2007 Elsevier Ltd. All rights reserved.
doi:10.1016/j.jpsychires.2007.04.002

Please cite this article in press as: Numata S et al., TGFBR2 gene expression and genetic association with schizophrenia, Journal of Psychiatric Research (2007), doi:10.1016/j.jpsychires.2007.04.002

phrenics no matter how taking or not taking antipsychotic medications (Cui et al., 2005). There are several studies that the tumor suppressor gene p53 (TP53), which is a key element in maintaining genomic stability and cell apoptosis, is associated with schizophrenia (Yang et al., 2004; Ni et al., 2005).

Transforming growth factor-β receptor 2 (TGFBR2) gene is a putative tumor suppressor gene implicated in several malignancies (e.g. colon cancer, gastric cancer, gliomas, etc.) (Markowitz et al., 1995; Myeroff et al., 1995; Izumoto et al., 1997), and recently has been to be associated with Marfan syndrome (Mizuguchi et al., 2004). There have been several reports of Marfan syndrome co-segregating with schizophrenia within families (Romano and Linares, 1987; Sirota et al., 1990), which suggest that some genetic resemblances may be shared between schizophrenia and Marfan syndrome. The TGFBR2 gene consists of seven exons and encodes the human TGF-β receptor, type II. This receptor belongs to the serine-threonine kinase family of cell surface receptors, which regulates several cellular processes, including proliferation, cell cycle arrest, apoptosis, differentiation and formation of extra cellular matrix (Annes et al., 2003; ten Dijke and Hill, 2004). TGFBR2 is expressed in the brain as well as other tissues and its locus lies at chromosome 3p22, which has been previously reported to be linked with schizophrenia (Lewis et al., 2003). These above findings imply that TGFBR2 gene may be involved in the pathogenesis of schizophrenia.

To investigate the pathological role of TGFBR2 gene to schizophrenia, we measured the TGFBR2 mRNA expression levels in the peripheral leukocytes of medication-free 19 schizophrenic patients, 25 major depressive patients and age- and sex-matched control subjects using a quantitative real time PCR method. In addition, we conducted a genetic case-control study of the TGFBR2 gene with schizophrenia in Japanese subjects (schizophrenics; $n = 279$, control subjects; $n = 279$).

2. Materials and methods

2.1. Subjects for analysis

All patients and control subjects were biologically unrelated Japanese. The diagnosis of schizophrenia and major depression was made by at least two experienced psychiatrists according to DSM-IV criteria (American Psychiatric Association, 1994). Clinical symptoms were evaluated by the Brief Psychiatric Rating Scale scores (BPRS) (Overall and Gorham, 1962) in schizophrenic patients when blood samples were taken. Age- and sex-matched controls were in good physical health without a history of any psychiatric or serious somatic diseases and taking any medication during the sample collection period. Probands who had first-degree relatives with psychiatric disorders were excluded from the control subjects.

Table 1a
Demographic data for medication-free schizophrenic patients studied in TGFBR2 mRNA expression analysis ($N = 19$)

	Age (y.o)	Gender	Age at onset (years)	BPRS score	Family history of Schizophrenia in first-degree relative
S1	25	M	22	64	+
S2	24	M	24	42	−
S3	24	M	24	31	−
S4	27	M	24	37	−
S5	36	M	36	34	−
S6	39	M	38	59	−
S7	27	M	26	58	−
S8	20	F	19	46	−
S9	23	F	23	48	−
S10	34	F	31	36	−
S11	47	F	47	30	−
S12	15	F	13	30	+
S13	26	F	21	100	−
S14	23	M	23	31	−
S15	28	M	25	63	−
S16	47	F	47	37	−
S17	37	F	21	36	−
S18	30	F	25	41	−
S19	45	F	43	36	+

The age (years old: y.o) represent the age of the subject when the leukocytes were drawn. M = male, F = female, + indicates that at least one of the first-degree relatives has schizophrenia.

For the measurement of expression levels of the TGFBR2 mRNA, the subjects consisted of 19 medication-free patients with schizophrenia (subject number S1–S19, Tables 1a and 1b) (14 first-episode and drug-naïve schizophrenic patients, 5 schizophrenic patients without antipsychotic treatment for at least two months; 9 males and 10 females, mean age: 30.4 ± 9.3), 19 age- and sex-matched controls for schizophrenic patients (9 males and 10 females, mean age: 30.6 ± 8.6), 25 medication-free patients with major depression (17 first-episode and drug-naïve depressive patients, 8 depressive patients without antidepressant treatment for at least two months; 9 males and 16 females, mean age: 39.8 ± 13.2) and 25 age- and sex-matched controls for depressive patients (9 males and 16 females, mean age: 40.9 ± 13.1). In addition, The TGFBR2 mRNA levels after antipsychotic treatment for several weeks were investigated in 13 out of 19 subjects (subject number S1–S13, Tables 1a and 1b, 7 males and 6 females, mean age: 28.2 ± 8.6) who were able to be followed up and compared with 13 age- and sex-matched controls (7 males and 6 females, mean age: 28.6 ± 7.5).

For the genetic studies, we used genomic DNA samples from 279 in-patients (189 male and 90 female; mean age: 51.3 ± 13.7 years) with schizophrenia from eleven psychiatric hospitals in the neighboring area of Tokushima Prefecture in Japan (population: about 820,000). Age- and sex-matched controls were selected from volunteers after assessing psychiatric problems (189 male and 90 female; mean age: 51.4 ± 12.0) for the association and haplotype-based case- control studies.

Please cite this article in press as: Numata S et al., TGFBR2 gene expression and genetic association with schizophrenia, Journal of Psychiatric Research (2007), doi:10.1016/j.jpsychires.2007.04.002

Table 1b
TGFBR2 mRNA expression in medication-free schizophrenic ($N = 19$) and control subjects ($N = 19$)

		Male ($N = 9$)	Female ($N = 10$)	Total ($N = 19$)
Schizophrenia (S1–S19)	Age	28.1 ± 5.6	32.4 ± 11.5	30.4 ± 9.3
	The TGFBR2 mRNA expression before treatment	Isoform A + isoform B		
		0.99 ± 0.23	1.11 ± 0.18	1.05 ± 0.20*
		Isoform B		
		1.00 ± 0.24	1.19 ± 0.34	1.11 ± 0.30*
Control	Age	27.6 ± 4.8	33.4 ± 10.4	30.6 ± 8.6
	The TGFBR2 mRNA expression	Isoform A + isoform B		
		0.79 ± 0.17	0.83 ± 0.16	0.81 ± 0.16
		Isoform B		
		0.78 ± 0.12	0.88 ± 0.16	0.83 ± 0.15

The mean TGPBR2 mRNA levels of medication-free schizophrenia patients were significantly higher than those of age-and sex-matched controls (isoform A + isoform B, $P < 0.001$, isoform B; $P = 0.003$, paired T-test). No correlation between TGFBR2 mRNA levels and baseline BPRS scores were observed (isoform A + isoform B; $P = 0.23$, isoform B; $P = 0.97$, Spearman's correlation efficient).

* $P < 0.01$, compared with the control group.

All subjects signed written informed consent to participate in the expression and genetic association studies approved by the institutional ethics committees.

3. Quantitative real-time PCR

Total RNA was extracted from the peripheral leukocytes using the PAXgene Blood RNA kit (Qiagen, Tokyo, Japan) according to the manufacturer's recommendations. One microgram of total RNA was used for cDNA synthesis by QuantiTect Reverse Transcription Kit (Qiagen, Japan) after assessing RNA quality and quantity with NanoDrop (NanoDrop Technologies, DE, USA). Expression of the TGFBR2 gene transcript was quantified by real-time PCR with the TaqMan Gene Expression Assay (Applied Biosystems, CA, USA). TGFBR2 gene has two splicing variants (isoform A, isoform B) (Lin et al., 1992; Nikawa, 1994). Suzuki et al. indicated that both isoforms of TGFBR2 gene mouse homolog are expressed in all tissues studied (Suzuki et al., 1994) and Hirai et al. showed that the isoform B is a major type of human TGFBR2 mRNA determined by RT-PCR (Hirai and Fujita, 1996). We measured the expression levels of isoform B separately as well as the transcript combinations of isoform A + isoform B using ABI probe/primers (Hs00559661_m1, Hs00947893_m1). GAPDH gene expression was used as an internal control and measurement of threshold cycle (Ct) was performed in triplicate. Data were collected and analyzed with Sequence Detector Software version 2.1 (Applied Biosystems) and the standard curve method. Relative gene expression was calculated as the ratio of TGFBR2 to GAPDH gene and the mean of the three replicate measures was assigned to each individual. Chronbach's alpha coefficient of three replicate measures was 0.980 and standard error of measurement was 0.122. The expression of the TGFBR2 mRNA in the peripheral leukocytes was not changed among blood samples collected at several points during the day time or over several weeks in the same control subject.

4. Genotyping

Genotyping was performed using commercially available TaqMan probes for TGFBR2 gene (C_29354774_10, C_29354775_10, C_27491740_10, C_1612565_10, C_11565984_20, C_1612508_10, C_11566050_10, C_8778140_10, C_25809090_10, C_15882489_10) with Applied Biosystems 7500 Fast Real Time PCR System according to the protocol recommended by the manufacturer (Applied Biosystems, CA, USA). We selected these 10 single nucleotide polymorphic (SNP) markers for genotyping from the public databases (dbSNP Home page) according to International Hap Map Project (http://www.hapmap.org/index.html.en). The heterozygocities of these 10 SNPs, rs7625858 (C/T), rs7648606 (C/T), rs3087465 (A/G), rs4522809 (C/T), rs12487185 (A/G), rs1864615 (A/G), rs3773652 (A/G), rs1367609 (A/C), rs3773663 (A/G) and rs2276767 (A/C) in Japanese population are reported as 0.23, 0.10, 0.18, 0.38, 0.37, 0.45, 0.48, 0.49, 0.42 and 0.09, respectively.

5. Statistical analysis

Statistical calculations were carried out using the SPSS Statistical Software Package 11.5 (SPSS, Tokyo, Japan). Expressional differences between patients and age-and sex-matched control subjects were calculated using the paired T-test after checking equal variances by Kolmogorov–Smirnov test. Changes before and after treatment were also analyzed with the paired T-test. Spearman correlation coefficients were used to evaluate the correlations between TGFBR2 mRNA levels and BPRS scores. Analysis of covariance (ANCOVA) was performed to determine the independent and combined effect of sex, diagnosis and age with the expression of TGFBR2 between groups. All significance levels were two-tailed. Allele and genotype frequencies of patients and control subjects were compared using Fisher's exact test. The SNPAlyze 3.2Pro software (DYNACOM, Japan) was used to estimate haplotype fre-

Please cite this article in press as: Numata S et al., TGFBR2 gene expression and genetic association with schizophrenia, Journal of Psychiatric Research (2007), doi:10.1016/j.jpsychires.2007.04.002

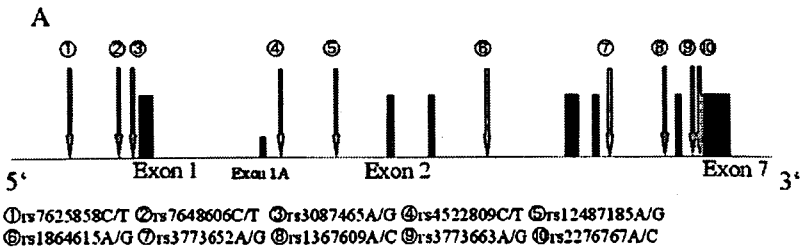


Fig. 1. Graphic representation of the TGFBR2 gene and the SNPs analyzed in the present study. Isoform B is major spliced variant without exon 1A. The amino acid sequence of isoform A contains an inset of 26 amino acids after Ser31, replacing Val132 of TGFBR2 isoform B.

quencies, LD, and permutation P values. Pair-wise linkage disequilibrium (LD) indices, D' and r2, were calculated in the control subjects. The criterion for significance was set at $P < 0.05$ for all tests. Data are presented as mean ± SD. Our sample size had a post hoc power of 0.81 to detect an effect size of $w = 0.22$ at the 0.05 significance level, as calculated by software program G Power (Erdfelder et al., 1996) (see Fig. 1).

6. Results

6.1. TGFBR2 mRNA expression in medication free schizophrenic and control subjects (Tables 1a and 1b)

Relative expression levels of TGFBR2 mRNA (isoform A + isoform B) in 19 medication-free patients were 1.05 ± 0.20, while 0.81 ± 0.16 in healthy volunteers, showing a statistical difference (paired T-test: $P < 0.001$, Kolmogorov–Smirnov test: $P = 0.200$, Fig. 2). No correlation between TGFBR2 mRNA levels and baseline BPRS scores were observed (Spearman's correlation efficient: $P = 0.23$). The same result was also obtained in the mRNA expression levels of TGFBR2 isoform B (data shown in Tables 1a and 1b).

6.2. TGFBR2 mRNA expression in schizophrenia after several weeks antipsychotic treatment (Tables 2a and 2b)

The TGFBR2 mRNA levels after antipsychotic treatment for several weeks were investigated in 13 subjects who were able to be followed up among 19 medication-free patients. Mean chlorpromazine-equivalent doses were 490.4 ± 510.1 mg/day and mean duration of treatment was 68.6 ± 23.9 days. BPRS scores were significantly improved after antipsychotic treatment for several weeks (at baseline: 43.3 ± 19.6, after treatment: 35.1 ± 13.4; paired T-test: $P = 0.002$, Kolmogorov–Smirnov test: $P = 0.200$) and the mean TGFBR2 mRNA levels (isoform A + isoform B) also showed a significant decrease toward healthy control levels after antipsychotic treatment (at baseline: 1.04 ± 0.18, after treatment: 0.88 ± 0.23; paired T-test: $P = 0.027$, Kolmogorov–Smirnov test: $P = 0.200$). The TGFBR2 mRNA levels after treatment were not different from controls' (paired T-test: $P = 0.14$). No correlation between TGFBR2 mRNA levels and BPRS scores after treatment were observed (Spearman's correlation efficient: $P = 0.37$). The changes of BPRS scores did not show significant correlation with the change of the mRNA levels (Spearman correlation efficient: $P = 0.86$).

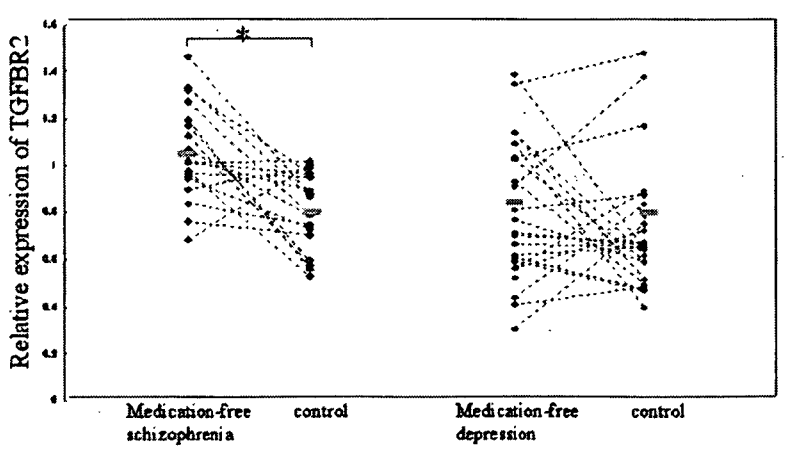


Fig. 2. Compared with the normal control group, the mean TGFBR2 mRNA level (isoform A + isoform B) in the leukocytes of medicaction-free schizophrenic patients ($N = 19$) was significantly higher than that of age- and sex-matched controls (patients: 1.05 ± 0.20, controls: 0.81 ± 0.16, paired T-test: $P < 0.001$). The mean TGFRBR2 mRNA level (isoform A + isoform B) in the leukocytes of medication-free major depressive patients ($N = 25$) showed no significant difference compared with sex- and age-matched controls (patients: 0.89 ± 0.31, controls: 0.84 ± 0.28, paired T-test: $P = 0.452$). *$P < 0.01$, compared with the control group.

Please cite this article in press as: Numata S et al., TGFBR2 gene expression and genetic association with schizophrenia, Journal of Psychiatric Research (2007), doi:10.1016/j.jpsychires.2007.04.002

The same result was also obtained in the mRNA expression levels of TGFBR2 isoform B (data shown in Tables 2a and 2b).

6.3. TGFBR2 mRNA expression in medication free major depression and control subjects

Relative expression levels of TGFBR2 mRNA (isoform A + isoform B) in 25 medication-free major depressive patients were 0.89 ± 0.31, while 0.84 ± 0.28 in healthy volunteers, showing no significant statistical difference (paired T-test: $P = 0.452$, Fig. 2). TGFBR2 mRNA expression levels of isoform B also showed the same result.

7. Genetic association analysis (Tables 3 and 4)

There were no significant deviations in all 10 SNPs from Hardy–Weinberg equilibrium in either patients or control subjects. Allele and genotype frequencies of the eight SNPs are shown in Table 4. There were no associations between these SNPs and schizophrenia neither in the allelic frequencies nor in the genotypic distributions. Permutation test of rs7625858–rs7648606 ($D' = 0.895$), rs7648606–rs3087465 ($D' = 0.866$) and rs3773663–rs2276767 ($D' = 0.945$) showed no significant difference in estimated frequencies of these haplotypes between the controls and patients (permutation $P = 0.19$, 0.27, 0.96, each).

Table 2a
Demographic data for schizophrenic patients after short-term antipsychotic treatment studied ill TGFBR2 mRNA expression analysis ($N = 13$)

	Age (y.o)	Gender	Duration of treatment (day)	Medication (at second point)	BPRS score
S1	25	M	90	Olz 10 mg	34
S2	24	M	134	Ris 3 mg	37
S3	24	M	54	Ris 3 mg	20
S4	27	M	55	Sulpiride 100 mg	27
S5	36	M	57	Olz 20 mg	23
S6	39	M	74	Olz 20 mg	36
S7	27	M	59	Olz 5 mg	47
S8	20	F	57	Ris3 mg, Lp25 mg	36
S9	23	F	71	Ris 2 mg	34
S10	34	F	85	Ris 2 mg	20
S11	47	F	47	Olz 15 mg	40
S12	15	F	44	Ris 2 mg	31
S13	26	F	65	Olz 20 mg, Ris 12 mg	71

Thirteen subjects (S1–S13) in Tables 2a and 2b were samples who were able to be followed up among 19 medication-free patients in Tables 1a and 1b. The age (years old: y.o) represent the age of the subject when the leukocytes were drawn. M = male, F = female, Olz: olanzapine, Ris: risperidone, LP: levomepromazine.

Table 2b
TGFBR2 mRNA expression in schizophrenics before treatment and after several weeks antipsychotic treatment ($N = 13$) and control subjects ($N = 13$)

		Male ($N = 7$)	Female ($N = 6$)	Total ($N = 13$)
Schizophrenia (S1–S13)	Age	28.9 ± 6.1	27.5 ± 11.5	28.2 ± 8.6
	The TGFBR2 mRNA expression before treatment	Isoform A + isoform B		
		1.00 ± 0.20	1.08 ± 0.16	1.04 ± 0.18*
		Isoform B		
		0.97 ± 0.21	1.13 ± 0.39	1.04 ± 0.30*
	The TGFBR2 mRNA expression after treatment	Isoform A + isoform B		
		0.75 ± 0.23	1.03 ± 0.10	0.88 ± 0.23
		Isoform B		
		0.61 ± 0.19	0.86 ± 0.17	0.72 ± 0.22
Control	Age	28.1 ± 5.2	29.2 ± 10.0	28.6 ± 7.5
	The TGFBR2 mRNA expression	Isoform A + isoform B		
		0.76 ± 0.18	0.77 ± 0.17	0.77 ± 0.17
		Isoform B		
		0.78 ± 0.14	0.82 ± 0.13	0.80 ± 0.13

BPRS scores were significantly improved after antipsychotic treatment for several weeks (at baseline: 43.3 ± 19.6, after treatment: 35.1 ± 13.4; paired T-test: $P = 0.002$).

The mean TGFBR2 mRNA levels showed a significant decrease toward healthy control levels after antipsychotic treatment (isoform A + isoform B; $P = 0.027$, isoform B; $P = 0.003$, paired T-test).

The TGFBR2 mRNA levels after treatment were not different from controls' (isoform A + isoform B; $P = 0.14$, isoform B; $P = 0.20$, paired T-test).

* $P < 0.05$, compared with the control group.

Please cite this article in press as: Numata S et al., TGFBR2 gene expression and genetic association with schizophrenia, Journal of Psychiatric Research (2007), doi:10.1016/j.jpsychires.2007.04.002

Table 3
Linkage disequilibrium (LD) indices (lower left are r2, upper right are D')

	rs 7625858	rs 7648606	rs 3087465	rs 4522809	rs 12487185	rs 1864615	rs 3773652	rs 1367609	rs 3773663	rs 2276767
rs 7625858	–	0.89465	0.58411	0.39018	0.38766	0.11919	0.00178	0.08145	0.06098	0.00053
rs 7648606	0.24556	–	0.8664	0.35761	0.31141	0.419	0.25127	0.18866	0.10999	0.59183
rs 3087465	0.30864	0.25458	–	0.0239	0.06625	0.00499	0.06456	0.03747	0.12032	0.03609
rs 4522809	0.01817	0.02333	0.00006	–	0.79095	0.69391	0.1183	0.18935	0.03031	0.81976
rs 12487185	0.02622	0.0121	0.00069	0.42727	–	0.76359	0.06257	0.1694	0.0435	0.49541
rs 1864615	0.00601	0.00907	0	0.13551	0.23876	–	0.04822	0.09866	0.07076	0.40741
rs 3773652	0	0.0034	0.00066	0.00943	0.00386	0.00224	–	0.14808	0.08763	0.43636
rs 1367609	0.00191	0.00315	0.00037	0.01748	0.02032	0.00568	0.01578	–	0.40153	0.87012
rs 3773663	0.00081	0.00081	0.00429	0.00034	0.0015	0.00259	0.00413	0.12234	–	0.94548
rs 2276767	0	0.00281	0.00053	0.02974	0.01588	0.02651	0.01238	0.08098	0.10863	–

Table 4
Genetic studies of TGFBR2 with schizophrenia in ease-control samples

Snp	Group	Genotype			n	Hardy–Weinberg P-value		Allele		P-value
rs7625858		T/T	C/T	C/C				T	C	
	sch	166	94	16	276	0.702	0.732	426	126	0.469
	cont	177	87	15	279	0.420		441	117	
rs7648606		T/T	C/T	C/C				T	C	
	sch	227	45	4	276	0.508	0.465	499	53	0.238
	cont	239	38	2	279	0.944		516	42	
rs3087465		A/A	A/G	G/G				A	G	
	sch	16	98	163	277	0.933	0.224	130	424	0.095
	cont	13	82	184	279	0.432		108	450	
rs4522809		T/T	T/C	C/C				T	C	
	sch	123	122	31	276	0.964	0.649	368	184	0.403
	cont	131	122	25	278	0.757		384	172	
rs12487185		A/A	A/G	G/G				A	G	
	sch	57	126	94	277	0.269	0.476	240	314	0.223
	cont	48	124	106	278	0.319		220	336	
rs1864615		A/A	A/G	G/G				A	G	
	sch	36	123	117	276	0.780	0.385	195	357	0.260
	cont	47	117	108	272	0.154		211	333	
rs3773652		A/A	A/G	G/G				A	G	
	sch	44	142	92	278	0.447	0.466	230	326	0.626
	cont	47	128	104	279	0.559		222	336	
rs1367609		A/A	C/A	C/C				A	C	
	sch	75	133	70	278	0.552	0.192	283	273	0.338
	cont	58	151	69	278	0.114		267	289	
rs3773663		A/A	A/G	G/G				A	G	
	sch	58	132	85	275	0.699	0.588	248	302	1.0
	cont	52	145	80	277	0.401		249	305	
rs2276767		A/A	A/C	C/C				A	C	
	sch	3	43	232	278	0.799	1.0	49	507	1.0
	cont	4	42	233	279	0.355		50	508	

sch, schizophrenia; cont, control subjects. P-values are calculated by Fisher's exact test.
There were no associations between these SNPs and schizophrenia neither in the allelic frequency not in the genotypic distributions.

8. Discussion

In the present study, relative expression levels of the TGFBR2 mRNA (isoform A + isoform B, isoform B) in both medication-free schizophrenic patients and major depressive patients were investigated. In addition, the association between 10 polymorphisms in the TGFBR2 locus and schizophrenia was investigated. To the best of our knowledge, this is the first study to investigate the role of TGFBR2 in the pathogenesis of schizophrenia.

First, our data showed that the mRNA expression level of TGFBR2 gene in the peripheral leukocytes was significantly higher in medication-free schizophrenics but not in medication-free depression. The results suggest that the expressional change of TGFBR2 gene in schizophrenia may be disease-specific and not due to non-specific stress from psychiatric condition. The BPRS scores were significantly improved after several week-antipsychotic treatment and the mean TGFBR2 mRNA levels showed a significant decrease toward healthy control levels after treatment. The

Please cite this article in press as: Numata S et al., TGFBR2 gene expression and genetic association with schizophrenia, Journal of Psychiatric Research (2007), doi:10.1016/j.jpsychires.2007.04.002

decrease of the TGFBR2 mRNA expression after treatment may be a consequence of pharmacological effects of antipsychotics or clinical improvement. These results suggest that altered expression of TGFBR2 mRNA in the peripheral leukocytes from schizophrenic patients may not be trait-oriented but state-related change. Be contrary to our anticipation, the mRNA expression level of TGFBR2 gene was not up-regulated in schizophrenia who took antipsychotic medications. TGFBR2 may be associated with reportedly low susceptibility to cancer in unmedicated but not medicated schizophrenia. Other tumor suppressor genes or oncogenes may have strong influence on tumor resistance associated with schizophrenia. In spite of the limited number of medication-free schizophrenic samples, the fact that altered mRNA expression of TGFBR2 gene in schizophrenia before treatment may have pathophysiological significance because peripheral lymphocytes could reflect the metabolism of brain cells (Gladkevich et al., 2004). Further expression study using human brain tissue is needed in order to reveal the pathological role of TGFBR2 gene to schizophrenia.

Second, we investigated the genetic association between TGFBR2 gene and schizophrenia in Japanese population. The TGFBR2 gene is located at 3p22, which has been previously reported to be linked with schizophrenia. However we did not find any association of 10 SNPs in TGFBR2 gene (rs7625858, rs7648606, rs3087465, rs4522809, rs12487185, rs1864615, rs3773652, rs1367609, rs3773663 and rs2276767) with schizophrenia. Haplotype analyses in the TGFBR2 gene did not reveal any significance, either. Further studies with denser polymorphisms and a larger sample set will be needed although our sample sizes were suitable for genetic comparison (power > 0.8).

In conclusion, our investigation revealed that the mean TGFBR2 mRNA levels (isoform A + isoform B, isoform B) in medication-free schizophrenic patients were significantly higher than those of age- and sex-matched controls and showed a significant decrease toward healthy control levels after antipsychotic treatment. There were no associations between the TGFBR2 gene and schizophrenia. We conclude that the TGFBR2 gene itself does not link to schizophrenia but that the TGFBR2 mRNA levels in the peripheral leukocytes may be a potential state marker for schizophrenia.

Acknowledgements

The authors thank all the volunteers who understood our study purpose and participated in this study and the physicians who helped us to take clinical data and blood samples in all the mental hospitals (Aizato Hospital, Akita Hospital, Fujii Hospital, Hosogi Unity Hospital, Jounan Hospital, Jousei Hospital, Kawauchi Hospital, Nankai Hospital, Sea Gull hospital, Taoka East Hospital, Tokushima prefectural central hospital and Yu-ai Hospital). The authors would like to thank Mrs. Akemi Okada and Mrs. Kumiko Kikuchi for their technical assistance. This work was supported by a Health and Labor Science Research Grant from the Japanese Ministry of Health, Labor and welfare, a Grant-in-Aid for Scientific Research from the Japanese Ministry of Education, Culture, Sports, Science and Technology and a Grant-in-Aid for Scientific Research from the 21st Century COE program, Human Nutritional Science on Stress Control, Tokushima, Japan.

References

American Psychiatric Association. Diagnostic and Statistical Manual of Mental Disorders. fourth ed. Washington, DC: American Psychiatric Press; 1994.

Annes JP, Munger JS, Rifkin DB. Making sense of latent TGF beta activation. Journal of Cell Science 2003;116:217–24.

Catts VS, Catts SV. Apotosis and schizophrenia: is the tumour suppressor gene, p53, a candiate susceptibility gene? Schizophrenia Research 2000;41:405–15.

Craddock N, O'Donovan MC, Owen MJ. The genetics of schizophrenia and bipolar disorder: dissecting psychosis. Journal of Medical Genetics 2005;42:193–204.

Cui DH, Jiang KD, Jiang SD, Xu YF, Yao H. The tumor suppressor adenomatous polyposis coli gene is associated with susceptibility to schizophrenia. Molecular Psychiatry 2005;10:669–77.

Erdfelder E, Faul F, Buchner A. GPOWER: a general power analysis progrm. Behavior Research Methods, Instruments, and Computers 1996;28:1–11.

Gladkevich A, Kauffman HF, Korf J. Lymphocytes as a neural probe: potential for studying psychiatric disorders. Progress in Neuropsychopharmacology and Biological Psychiatry 2004;28:559–76.

Grinshpoon A, Barchana M, Ponizovsky A, Lipshitz I, Nahon D, Tal O, et al. Cancer in schizophrenia: is the risk higher or lower? Schizophrenia Research 2005;73:333–41.

Hirai R, Fujita T. A human transforming growth factor-beta type II receptor that contains an insertion in the extracellular domain. Experimental Cell Research 1996;223:135–41.

Izumoto S, Arita N, Ohnishi T, Hiraga S, Taki T, Tomita N, et al. Microsatellite instability and mutated tapeIItransforming growth factor-βreceptor gene in gliomas. Cancer Letters 1997;112:251–6.

Lewis CM, Levinson DF, Wise LH, DeLisi LE, Straub RE, Hovatta I, et al. Genome scan meta-analysis of schizophrenia and bipolar disorder, Part II: schizophrenia. American Journal of Human Genetics 2003;73:34–48.

Lin HY, Wang XF, Ng-Eaton E, Weinberg RA, Lodish HF. Expression cloning of the TGF-beta type II receptor, a functional transmembrane serine/threonine kinase. Cell 1992;68:775–85.

Markowitz S, Wang J, Myeroff L, Parsons R, Sun L, Lutterbaugh J, et al. Inactivation of the type II TGF-β receptor in colon cancer cells with microsatellite instability. Science 1995;268:1336–8.

Mizuguchi T, Collod-Beroud G, Akiyama T, Abifadel M, Harada N, Morisaki T, et al. Heterozygous TGFBR2 mutations in Marfan syndrome. Nature Genetics 2004;36:855–60.

Myeroff LL, Parsons R, Kim SJ, Hedrick L, Cho KR, Orth K, et al. A transforming growth factorβ-receptor type II gene mutation common in colon and gastric but rare in endometrial cancers with microsatellite instability. Cancer Research 1995;55:5545–7.

Ni X, Trakalo J, Valente J, Azevedo MH, Pato MT, Pato CN, et al. Human p53 tumor suppressor gene (TP53) and schizophrenia: case-control and family studies. Neuroscience Letters 2005;388:173–8.

Nikawa J. A cDNA encoding the human transforming growth factor beta receptor suppresses the growth defect of a yeast mutant. Gene 1994;149:367–72.

Overall JF, Gorham DR. The brief psychiatric rating scale. Psychological Reports 1962;10:799–812.

Romano J, Linares RL. Marfan syndrome and schizophrenia: a case report (letter). Archives of General Psychiatry 1987;44:190–2.

Please cite this article in press as: Numata S et al., TGFBR2 gene expression and genetic association with schizophrenia, Journal of Psychiatric Research (2007), doi:10.1016/j.jpsychires.2007.04.002

Sirota P, Frydman M, Sirita L. Schizophrenia and Marfan syndrome. British Journal of Psychiatry 1990;157:433–6.

Suzuki A, Shioda N, Maeda M, Tada M, Ueno N. Cloning of an isoform of mouse TGF-beta type II receptor gene. FEBS Letters 1994;355:19–22.

ten Dijke P, Hill CS. New insights into TGF-beta-Smad signaling. Trends in Biochemical Sciences 2004;29:265–73.

Yang Y, Xiao Z, Chen W, Sang H, Guan Y, Peng Y, et al. Tumor suppressor gene TP53 is genetically associated with schizophrenia in the Chinese population. Neuroscience Letters 2004;369:126–31.

Please cite this article in press as: Numata S et al., TGFBR2 gene expression and genetic association with schizophrenia, Journal of Psychiatric Research (2007), doi:10.1016/j.jpsychires.2007.04.002