the association in immortalized lymphoblasts derived from 42 patients with schizophrenia and 44 healthy controls ($z = 3.09$, $P = 0.002$). The expression of the high-risk TG haplotype was significantly lower than the protective TA haplotype. The expression was lower in patients with schizophrenia than in controls; however, this difference was not statistically significant. This study provides further evidence of the association of the *NRGN* gene with schizophrenia, and our results suggest that there is a link between the TG haplotype of rs12807809–rs12278912, decreased expression of *NRGN* and risk of developing schizophrenia. © 2012 Wiley Periodicals, Inc.

Key words: schizophrenia; *neurogranin* (*NRGN*); single nucleotide polymorphism (SNP); genome-wide association study (GWAS); gene expression

INTRODUCTION

Schizophrenia is a common and complex psychiatric disease with strong genetic components. Schizophrenia has an estimated heritability of approximately 80% [Cardno and Gottesman, 2000; Tsuang, 2000], and many genes have been implicated in the pathogenesis of schizophrenia [Sun et al., 2008].

Genome-wide association studies (GWAS) of single nucleotide polymorphisms (SNPs) investigate thousands of DNA samples from patients and controls, and these studies are a powerful tool for identifying common risk factors in complex diseases. Stefansson et al. [2009] combined the samples (from 12,945 patients with schizophrenia and 34,591 controls) from three large GWAS (the SGENE-plus, the International Schizophrenia Consortium and the Molecular Genetics of Schizophrenia GWAS) and conducted follow-up studies in 4,999 patients and 15,555 controls from four sets of samples from Europe, including from the Netherlands, Denmark, Germany, Hungary, Norway, Russia, Sweden, Finland, and Spain. The authors detected several significant association signals. Seven markers gave *P* values smaller than the genome-wide significance threshold of approximately 1.6×10^{-7} in the combined samples: five markers, rs6913660, rs13219354, rs6932590, rs13211507, and rs3131296, which spanned the major histocompatibility complex (MHC) region on chromosome 6p21.3-22.1; a marker, rs12807809, located 3,457 bases upstream of the *neurogranin* (*NRGN*) gene on chromosome 11q24.2; and a marker, rs9960767, in intron 4 of the transcription factor 4 (*TCF4*) gene on chromosome 18q21.2. Of the seven SNPs, four SNPs, rs6913660, rs13219354, rs13211507, and rs9960767, were not polymorphic in the HapMap Japanese in Tokyo (JPT) samples. The minor allele frequencies (MAFs) for two SNPs, rs6932590 and rs3131296, were less than 5%. Because only one marker, rs12807809, in the *NRGN* gene was a common SNP in the HapMap JPT samples (MAF greater than 5%), we focused on this SNP and the *NRGN* gene in the present study.

The *NRGN* gene is the human homolog of the neuron-specific rat RC3/*neurogranin* gene. *NRGN* encodes a postsynaptic protein kinase substrate that binds calmodulin (CaM) in the absence of calcium and has been implicated in dendritic spine formation and synaptic plasticity [Baudier et al., 1991]. NRGN plays an important role in the Ca^{2+}–CaM signaling pathway [Hayashi, 2009]. Ca^{2+} influx-induced oxidation of NRGN leads to the postsynaptic activation of CaM-dependent protein kinase II (CaMKII) by CaM, which is associated with strengthened *N*-methyl-D-aspartate (NMDA) receptor signaling [Li et al., 1999]. Reduced NRGN activity may mediate the effects of NMDA hypofunction implicated in the pathophysiology of schizophrenia.

The *NRGN* gene spans 7.3 kb of genomic DNA and contains four exons [Martinez de Arrieta et al., 1997]. Part of exon 1 and exon 2 encode a 78-amino-acid protein, and exons 3 and 4 contain untranslated sequences. A thyroid hormone response element (TRE) has been identified in intron 1 [Martinez de Arrieta et al., 1999]. An association between the *NRGN* gene and schizophrenia has previously been reported in a small population of male Portuguese and Brazilians [Ruano et al., 2008], although the associated SNP in the study, rs7113041, was not tightly correlated with the genome-wide supported SNP, rs12807809 (HapMap CEU $r^2 = 0.07$, JPT $r^2 = 0.01$). In addition, two separate studies reported no association between the genetic variants of *NRGN* and schizophrenia in Bulgarian [Betcheva et al., 2009] and Chinese populations [Li et al., 2010]. The genome-wide supported SNP and other SNPs in the *NRGN* gene were not genotyped in the GWAS of schizophrenia in Japanese populations because of a difference in the genotyping chips used among the separate GWAS, which the Illumina HumanHap 300 or 550 BeadChips, Affymetrix Genome-Wide Human SNP Array 5.0 and Affymetrix GeneChip Mapping 100 K microarrays [Stefansson et al., 2009; Ikeda et al., 2011; Yamada et al., 2011] were used. Here, we first investigated the association between the *NRGN* gene and schizophrenia in a Japanese population using a gene-based approach to determine whether rs12807809 is the most strongly associated variant for schizophrenia near the *NRGN* gene. Second, we examined whether the associated haplotype of *NRGN* influenced *NRGN* expression in immortalized lymphoblasts derived from the HapMap JPT samples and our Japanese case–control samples.

MATERIALS AND METHODS

Subjects

Subjects for the genetic association analysis included 2,019 unrelated patients with schizophrenia (54.5% males, with a mean age ± SD of 44.7 ± 15.1 years) and 2,579 unrelated healthy controls (49.4% males, 45.4 ± 19.4 years). The mean age did not differ significantly between cases and controls ($P = 0.24$); however, the male to female ratio of the patients was significantly higher than in the controls ($P < 0.05$). Age and sex-matched subjects for *NRGN* expression analysis consisted of 42 patients with schizophrenia (58.1%, 38.4 ± 11.2 years) and 44 healthy subjects (56.8% males, 38.0 ± 11.4 years). These subjects were included in the genetic association analysis. All subjects used in both analyses were biologically unrelated, of Japanese ethnicity and were recruited from four geographical regions in Japan: Osaka, Aichi, Tokushima, and Tokyo [Yamaguchi-Kabata et al., 2008; Ohi et al., 2009]. Cases were recruited from outpatient and inpatient facilities at university hospitals and psychiatric hospitals. Each subject with schizophrenia had been diagnosed by at least two trained psychiatrists based on an unstructured clinical interview; diagnoses were made based on the

criteria of the fourth edition of the Diagnostic and Statistical Manual of Mental Disorders (DSM-IV). Controls were recruited through local advertisements. Psychiatrically healthy controls were evaluated using unstructured interviews to exclude individuals who had current or past contact with psychiatric services. Written informed consent was obtained for all subjects after the procedures had been fully explained. This study was carried out in accordance with the World Medical Association's Declaration of Helsinki and approved by the Research Ethical Committee of Osaka University, Fujita Health University, Nagoya University, Tokushima University and Juntendo University.

SNP Selection and SNP Genotyping

This study was designed to examine the association between the *NRGN* gene and schizophrenia by selectively tagging SNPs in the *NRGN* gene and flanking regions (±5 kb). We selected five tagging SNPs using the TAGGER algorithm (Paul de Bakker, http://www.broad.mit.edu/mpg/tagger) with the criteria of r^2 greater than 0.80 in "pair-wise tagging only" mode and an MAF greater than 5%, which was implemented in Haploview 4.2 using HapMap data release 27 Phase II + III, Feb 2009, on NCBI B36 assembly, dbSNP b126 [Japanese in Tokyo (JPT), Chr 11: 124,109,952.124,127,307]. The five tagging SNPs were rs1939214, rs12807809, rs12278912, rs2075713, and rs11219769. Markers are shown in Table I; orientation and alleles are reported on the genomic plus strand (rs12807809 is reported as T/C, as has been reported in previous GWAS [Stefansson et al., 2009]). Venous blood was collected from the subjects and genomic DNA was extracted from whole blood according to standard procedures. The SNPs were genotyped using the TaqMan 5′-exonuclease allelic discrimination assay (Applied Biosystems, Foster City, CA) as previously described [Hashimoto et al., 2006, 2007]. Detailed information on the PCR conditions is available upon request. Genotyping call rates were 98.9% (rs1939214), 98.5% (rs12807809), 99.3% (rs12278912), 99.3% (rs2075713), and 99.5% (rs11219769). No deviation from Hardy–Weinberg equilibrium (HWE) in the examined SNPs was detected in the patients with schizophrenia or healthy controls ($P > 0.05$). The positions of the five SNPs analyzed in the present study are shown in Figure 1.

Quantitative Measurement of *NRGN* Gene Expression

Isolation and immortalization procedures of lymphocytes from blood using the Epstein–Barr virus (EBV) were performed by SRL of Tokyo, Japan. Immortalized, patient-derived lymphocytes were grown in culture media supplemented with 20% fetal bovine serum. Total RNA was extracted from cell pellets using the RNeasy Mini Kit (Qiagen K.K., Tokyo, Japan). The total yield of RNA was determined by absorbance at 260 nm, and the quality of the RNA was determined using agarose gel electrophoresis.

According to the manufacturer's protocol, total RNA was treated with DNase to remove contaminating genomic DNA using DNase Treatment and Removal Reagents (Ambion, Austin, TX). Total RNA (10 µg) treated with DNase was used in a 50-µl reverse transcriptase reaction to synthesize cDNA with the SuperScript

TABLE I. Genotype and Allele Distributions for SNPs in the *NRGN* Gene Between Patients With Schizophrenia and Controls in a Japanese Population

Marker				SCZ (n = 2019)			CON (n = 2579)			Genotypic	MAF		Allelic	
SNP IDs (M)	Position[a]	M/m[b]	Gene	M/M	M/m	m/m	M/M	M/m	m/m	*P*-value (χ^2)	SCZ	CON	*P*-value (χ^2)	OR (95% CI)
rs1939214 (A)	124110500	A/G	5′	0.67	0.30	0.04	0.66	0.30	0.04	0.51 (1.3)	0.19	0.19	0.29 (1.1)	1.06 (0.95–1.18)
rs12807809 (T)[c]	124111495	T/C	5′	0.58	0.35	0.07	0.56	0.37	0.07	0.44 (1.6)	0.25	0.26	0.25 (1.3)	1.06 (0.96–1.16)
rs12278912 (G)[d]	124117369	G/A	Intron1	0.61	0.34	0.05	0.59	0.35	0.06	0.13 (4.1)	0.22	0.23	0.057 (3.6)	1.10 (1.00–1.22)
rs2075713 (A)	124123149	A/C	3′	0.65	0.31	0.04	0.62	0.33	0.05	0.17 (3.5)	0.20	0.21	0.057 (3.6)	1.10 (1.00–1.22)
rs11219769 (G)	124125357	G/T	3′	0.57	0.37	0.06	0.55	0.38	0.07	0.24 (2.8)	0.25	0.26	0.09 (2.8)	1.09 (0.99–1.19)

SCZ, patients with schizophrenia; CON, healthy controls; M, major allele; m, minor allele; MAF, minor allele frequency; OR, odds ratio.
[a]db SNP build 129.
[b]The first alleles shown are major alleles. All the alleles are represented according to the plus strand DNA sequence.
[c]The genome-wide supported SNP for schizophrenia [Stefansson et al., 2009].
[d]Because a high linkage disequilibrium between rs12278912 and rs7113041 [Ruano et al., 2008] was found in the HapMap JPT samples ($r^2 = 0.93$), rs12278912 was selected as the tagging SNP by the TAGGER program.

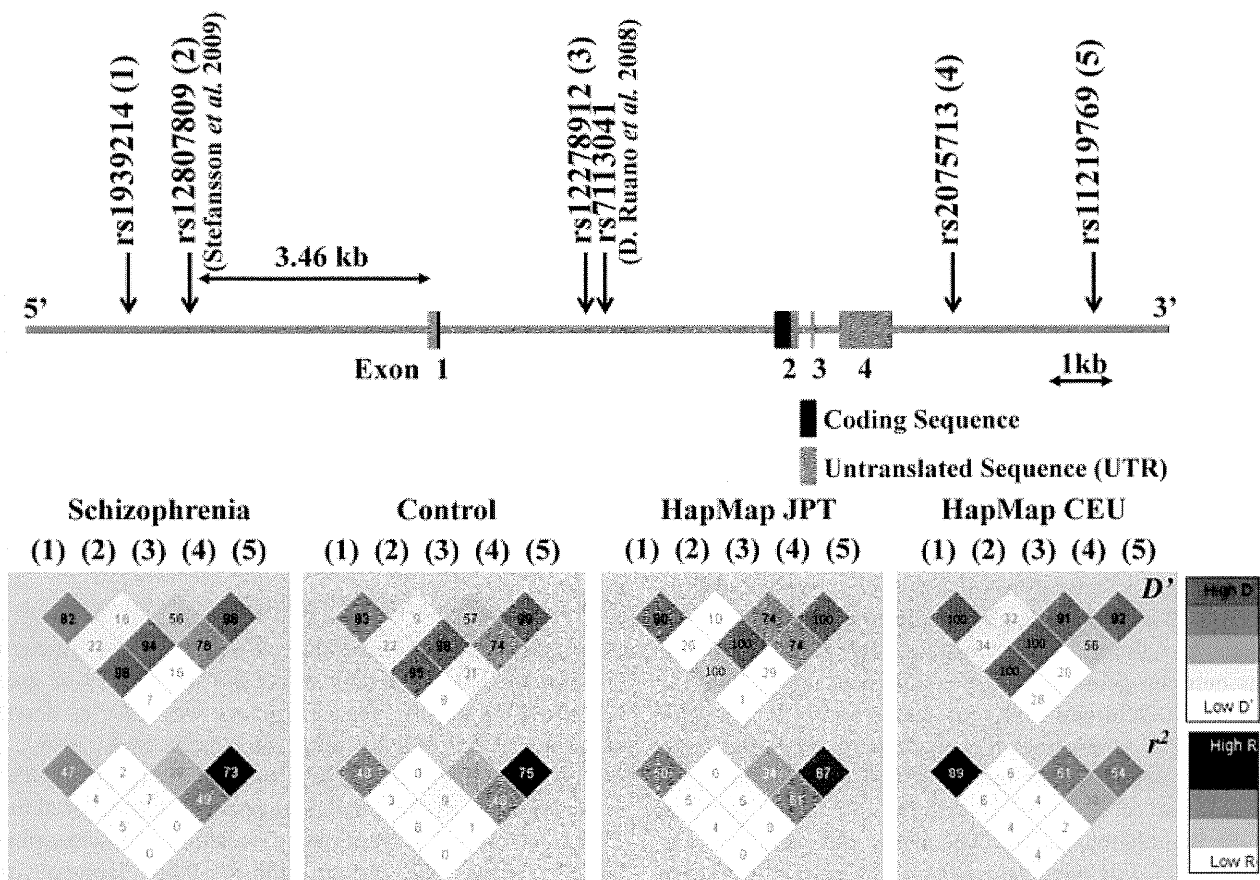

FIG. 1. The genomic structure of *NRGN*, including the locations of the five tagging SNPs studied and linkage disequilibrium of these SNPs in the patient, control, HapMap JPT, and CEU groups. Based on an entry in the Entrez Gene database (National Center for Biotechnology Information), the genomic structure of *NRGN* is shown above. The locations of the SNPs analyzed in this study are indicated by arrows, with numbers indicated in parentheses. The numbers indicated in parentheses refer to the numbering of the SNPs in the linkage disequilibrium (LD) diagram. The distances of exons–introns and intermarkers are drawn to scale. The LDs between pairwise SNPs are shown using the D′ (upper) and r^2 (lower) values at the bottom of the map of the gene structure separately for cases, controls, the HapMap JPT samples and the HapMap CEU samples. High levels of LD are represented by black (D′ and r^2) coloring, with increasing color intensity from 0 to 100, as shown by color bars.

First-Strand Synthesis System for RT-PCR (Invitrogen, Carlsbad, CA) according to the manufacturer's protocol. Detailed information on the PCR conditions is available upon request.

To measure mRNA expression levels of housekeeping (β-*actin*) and *NRGN* genes, we used the Pre-Developed TaqMan Assay Reagent kit (Applied Biosystems). Primer information (gene name: assay ID, transcript ID, target region) is as follows; *NRGN*: Hs00382922_m1, NM_001126181.1 and NM_006176.2, Exon1-2; β-actin: 4326315E, NM_001101, no region indicated (Applied Biosystems). Expression levels of these genes were measured by quantitative real-time reverse transcriptase polymerase chain reaction (qRT-PCR) using an ABI Prism 7900 Sequence Detection System (Applied Biosystems) with a 384-well format as previously described [Yamamori et al., 2011; Yasuda et al., 2011]. PCR data were obtained using Sequence Detector software (SDS version 2.1; Applied Biosystems) and quantified using a standard curve. This software plotted the real-time fluorescence intensity and selected the threshold within the linear phase of the amplicon profile. The software plotted a standard curve of the cycle at threshold C_t, which is where the fluorescence generated within a reaction crossed the threshold, versus the quantity of RNA. All samples were measured using a single plate per target gene, and their C_t values were in the linear range of the standard curve. The quantity of each sample was predicted by C_t values. The qRT-PCR reaction was performed in triplicate, and the expression level of the gene was taken as the average of three independent measurements. Standard curves were obtained using serial dilutions (1:4) of pooled complementary DNA prepared from 300 ng total RNA derived from immortalized lymphocytes. The standard curves of β-*actin* and *NRGN* showed that these genes were expressed in immortalized lymphocytes. In each experiment for β-*actin* and *NRGN*, the R^2 value of the standard curve was >0.99, and no-template control assays resulted in no detectable signal. The individual expression levels of the *NRGN* gene were normalized to the housekeeping gene (raw target gene expression level divided by raw housekeeping gene expression level) and were used for statistical analysis.

Haplotype Associated With *NRGN* Expression (eQTL)

To identify whether the haplotypes in *NRGN* associated with schizophrenia may be expression quantitative trait loci (eQTL), we analyzed *NRGN* expression in two datasets of lymphoblast-derived HapMap JPT samples and in the Japanese case–control samples. For the HapMap JPT samples, we extracted genotypes and *NRGN* lymphoblastoid expression data from the HapMap JPT samples ($n = 45$) deposited in GeneVar (http://www.sanger.ac.uk/humgen/genevar/ [Stranger et al., 2007]). For the Japanese case–control samples, we used our genotypes and *NRGN* lymphoblastoid expression data obtained using the method described above.

Statistical Analyses

We performed power calculations using the Power Calculator for Two-Stage Association Studies (http://www.sph.umich.edu/csg/abecasis/CaTS/ [Skol et al., 2006]). The power estimate was based on an allele frequency of 0.83 at rs12807809, an odds ratio of 1.19, which was indicated by Stefansson et al. [2009], a prevalence of 0.01, and an alpha level of 0.05 using a multiplicative model.

Differences in clinical characteristics between patients and controls or between genotypes were analyzed using χ^2 tests for sex and the Mann–Whitney *U*-test for age using PASW Statistics 18.0 software (SPSS Japan, Inc., Tokyo, Japan). Deviation from HWE was tested separately in test cases and controls using χ^2 tests for goodness of fit using SNPAlyze V5.1.1 Pro software (DYNACOM, Yokohama, Japan). The allelic and genotypic distributions of *NRGN* polymorphisms between patients and controls were analyzed using χ^2 tests with SNPAlyze V5.1.1 Pro software. The number of effective independent SNPs assayed was estimated to correct for multiple testing by the spectral decomposition method of Nyholt using the SNPSpD software [Nyholt, 2004]. The effective number of independent marker loci was 4.13 and corrected *P*-value for allelic and genotypic associations was set at $P < 0.012$. Pairwise linkage disequilibrium (LD) analyses expressed by D′ and r^2 were applied to detect the intermarker relationships in each group using Haploview 4.2 software (http://www.broad.mit.edu/mpg/haploview/contact.php). Haplotype frequencies were estimated using the method of maximum likelihood with genotyping data using the expectation–maximization (EM) algorithm from SNPAlyze V5.1.1 Pro software. Rare haplotypes detected in less than 3% of patients and controls were excluded from the haplotypic association analysis, as previously described [Ohi et al., 2009, 2010]. We performed 10,000 permutations for significance tests to determine empirical significance using a 2×2 contingency table approach. We used a 2- to 5-window fashion analysis. Since Bonferroni correction for multiple testing is considered to be too conservative to apply to genetic association analyses [Nyholt, 2001], method of Nyholt [Nyholt, 2004] for allelic and genotypic associations and permutation tests [Dudbridge, 2003] for haplotypic associations are considered to be appropriate for these analyses.

The difference in expression levels between Japanese patients with schizophrenia and controls was analyzed using linear regression in PASW Statistics 18.0 software. Age and sex, which may influence gene expression, were corrected for in the expression analysis. HPlus (http://qge.fhcrc.org/hplus) is a software application for estimating haplotype frequencies and inferring individual haplotypes based on EM and progressive ligation (PL) algorithms [Li et al., 2003], and most significantly assessing haplotypic associations with various types of phenotypes using linear regression. Differences of expression levels among haplotypes were analyzed using linear regression in HPlus software. Each genotype was treated as the number of major alleles (0, 1, and 2) in the expression analysis. For the joint haplotype analysis in HPlus software, each haplotype was tested against the reference haplotype (equal to most frequent haplotype) using linear regression. As age and sex were not available for the HapMap samples, these confounding factors were not corrected for in the expression analysis. Expression levels in Japanese cases, control samples and in the combined samples were corrected for age and sex in the analyses. We applied a Bonferroni correction in expression analysis (three tests). The significance level for statistical tests was set at two-tailed $P < 0.05$.

RESULTS

Genetic Association Analysis

Our study size of 2,019 cases and 2,579 controls had sufficient power (>80%) to detect a genetic effect at ORs of 1.19 or greater for rs12807809 when the allele frequency was 0.83, as described in previous GWAS (SGENE-plus) [Stefansson et al., 2009].

The genotype and allele frequencies of five tagging SNPs located in the *NRGN* gene and flanking regions are summarized in Table I. There was no allelic or genotypic association with schizophrenia for any of the five SNPs (uncorrected $P > 0.05$). However, nominal differences in allele frequencies between patients and controls were observed in rs12278912 ($\chi^2 = 3.6$, $P = 0.057$, corrected $P = 0.24$) and rs2075713 ($\chi^2 = 3.6$, $P = 0.057$, corrected $P = 0.24$). The major allele frequencies of both SNPs were higher in patients than in controls. Consistent with previous GWAS [Stefansson et al., 2009], the frequency of the major T allele of rs12807809 was higher in patients (75.4%) than in controls (74.4%) in our Japanese population, although the results did not reach statistical significance [$\chi^2 = 1.3$, $P = 0.25$, OR (95% confidence interval (CI)) = 1.06 (0.96–1.16)].

We focused on haplotypic association between patients with schizophrenia and healthy subjects using a 2- to 5-window fashion analysis. Haplotype analysis showed a significant association with schizophrenia (rs12807809–rs12278912, $\chi^2 = 13.1$, global $P = 0.0042$) (Supplementary Table I). The frequency of the major TG haplotype of rs12807809–rs12278912 was higher in patients (62%) than in controls (58%) [$\chi^2 = 9.4$, $P = 0.0019$, OR (95% CI) = 1.14 (1.05–1.24)] (Table II). On the other hand, the frequency of the TA haplotype of rs12807809–rs12278912 was lower in patients (14%) than in controls (16%) [$\chi^2 = 7.3$, $P = 0.0053$, OR (95% CI) = 0.85 (0.76–0.96)] (Table II). There was no haplotypic association with schizophrenia for any other haplotypes. These findings suggest that the major TG haplotype of rs12807809–rs12278912 may be related to an increased risk of schizophrenia, and the TA haplotype may have a protective role against the susceptibility to schizophrenia. These results of allelic, genotypic, or haplotypic associations were not affected by excluding 86 samples used for expression analyses (data not shown).

TABLE II. Differences in the rs12807809–rs12278912 Haplotype Between Patients With Schizophrenia and Healthy Subjects

Haplotype	Frequency		Individual P (χ^2)	OR (95%CI)	Global P (χ^2)
	Patients	Controls			
rs12807809[a]–rs12278912[b]					**<u>0.0042 (13.1)</u>**
TG	0.62	0.58	**<u>0.0019 (9.4)</u>**	1.14 (1.05–1.24)	
CG	0.17	0.18	0.07 (3.4)	0.90 (0.81–1.01)	
TA	0.14	0.16	**<u>0.0053 (7.3)</u>**	0.85 (0.76–0.96)	
CA	0.08	0.08	0.57 (0.3)	1.05 (0.90–1.22)	

Significant P values are shown as bold-faced and underlined type.
[a]The genome-wide supported SNP for schizophrenia [Stefansson et al., 2009].
[b]Because a high linkage disequilibrium between rs12278912 and rs7113041 [Ruano et al., 2008] was found in the HapMap JPT samples ($r^2 = 0.93$), rs12278912 was selected as the tagging SNP by the TAGGER program.

The LD relationships between the markers are provided in Figure 1. The LD pattern observed in our controls was similar to our patients and the JPT HapMap samples; however, it was different from that of the CEU HapMap samples. The strengths of the LD patterns of rs1939214–rs12807809 and rs12278912–rs2075713–rs11219769 were different between Japanese populations and the CEU HapMap samples. The low LD pattern of rs12807809–rs12278912 was similar among the groups ($D' < 0.50$, $r^2 < 0.10$).

NRGN Gene Expression Analysis

The *NRGN* expression level was lower in patients with schizophrenia (n = 42, mean ± SD, 0.86 ± 0.58) than in controls (n = 44, 1.00 ± 0.75). However, the results did not reach statistical significance (r = −0.14, beta = −0.11, SE = 0.14, t = −0.97, P = 0.34).

Based on the results from the genetic association analysis, we investigated whether the rs12807809–rs12278912 haplotype of the *NRGN* gene was an eQTL in two datasets. The rs12807809–rs12278912 haplotype related to schizophrenia was significantly associated with *NRGN* expression in healthy HapMap JPT samples. The *NRGN* gene expression of the high-risk TG haplotype of rs12807809–rs12278912 was significantly lower than that of the protective TA haplotype (z = 2.69, P = 0.007). We confirmed that the rs12807809–rs12278912 haplotype was significantly associated with *NRGN* expression normalized to the β-*actin* expression in the controls and combined samples (Fig. 2 and Table III, control samples: z = 2.30, P = 0.021, combined samples: z = 3.09, P = 0.002). The association occurred in the same direction among the HapMap JPT, control, and combined samples. In case samples, the expression level of rs12807809–rs12278912 was lower in samples with the high-risk TG haplotype than in those with the protective TA haplotype, although the result did not reach statistical significance (z = 1.49, P = 0.14). The association in the HapMap JPT and combined samples remained significant after correction for multiple tests (HapMap JPT samples: corrected P = 0.021, combined samples: corrected P = 0.006). However, there was no significant association after applying the correction in control samples (corrected P = 0.063).

DISCUSSION

In this study, we provided evidence that haplotypes, including the genome-wide-screen-supported SNP of the *NRGN* gene, were associated with an increased risk of schizophrenia. Our in silico analysis showed that the high-risk rs12807809–rs12278912 haplotype of the *NRGN* gene may be associated with a low expression level of the *NRGN* gene in lymphoblasts derived from the HapMap JPT samples. We confirmed the association between the haplotype and *NRGN* expression in the combined case–control samples. Our results suggest that the schizophrenia-associated haplotype at the

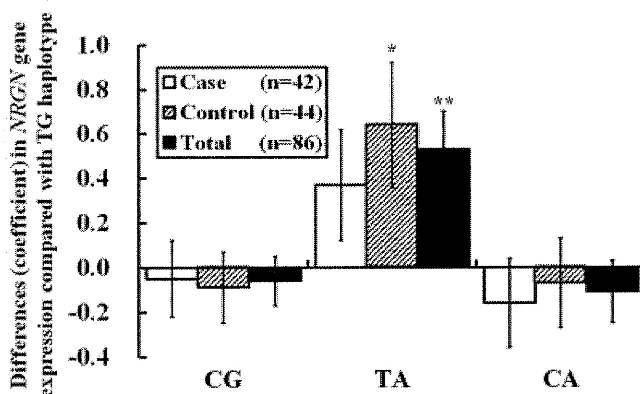

FIG. 2. The association between the rs12807809–rs12278912 haplotype of the *NRGN* gene and *NRGN* expression in lymphoblasts. Expression of the protective TA haplotype of rs12807809–rs12278912 was significantly higher than that of the high-risk TG haplotype in controls and combined case–control samples. The error bars represent standard errors of the coefficient. Estimated frequencies of each haplotype were as follows—TG haplotype: Case, 69%; Control, 61%; Total, 65%; CG haplotype: Case, 16%; Control, 20%; Total, 18%; TA haplotype: Case, 7%; Control, 11%; Total, 9%; CA haplotype: Case, 8%; Control, 9%; Total, 8%. *P < 0.05, **P < 0.01.

NRGN gene may be a functional variant, and the results support an association between the *NRGN* gene and schizophrenia.

This report is the first investigation of the association of tagging SNPs and haplotypes covering the *NRGN* gene with schizophrenia. To our knowledge, five genetic studies have investigated whether the *NRGN* gene is implicated in schizophrenia. A genome-wide linkage study has shown that the chromosomal region 11q23.3-24 including the *NRGN* gene is linked to schizophrenia in British and Icelandic populations [Gurling et al., 2001]. Subsequently, an association study determined that rs7113041, which displays high LD with rs12278912 and is located on intron 1 in the *NRGN* gene, is related to the risk of developing schizophrenia in male subjects of Portuguese origin [Ruano et al., 2008]. In addition, three GWAS and follow-up studies have shown that rs12807809 is associated with schizophrenia in large European samples [Stefansson et al., 2009]. However, two studies reported no association between *NRGN* and schizophrenia in Bulgarian or Chinese populations [Betcheva et al., 2009; Li et al., 2010]. In the present study, we determined that the rs12807809–rs12278912 haplotype is associated with an increased risk of schizophrenia in a Japanese population. However, there were no significant associations between any SNP, including rs12807809 and rs12278912, and schizophrenia in the population. The inconsistency of association among the previous studies and the present study might result from ethnic differences or type I or II errors for the different sample sizes: Portuguese, 315 cases, 295 controls and 73 trios [Ruano et al., 2008]; European Caucasian, 12,945 cases and 34,591 controls [Stefansson et al., 2009]; Japanese, 2,019 cases and 2,579 controls (present study); Bulgarian, 185 cases and 184 controls [Betcheva et al., 2009]; and Chinese, 2,496 cases and 5,184 controls [Li et al., 2010]. In addition, the SNPs investigated in each study were different. Ruano et al. [2008] and Betcheva et al. [2009] examined rs7113041, which has high LD with rs12278912 but not with rs12807809, whereas Stefansson et al. [2009] and Li et al. [2010] examined rs12807809 but not rs12278912. However, none of these studies examined haplotypes for the *NRGN* gene. Because the rs12807809–rs12278912 haplotype may be the most significant genetic variant in this region, further study is required to confirm the association between the rs12807809–rs12278912 haplotype and schizophrenia in other populations.

Differences in the relative *NRGN* expression levels between patients with schizophrenia and healthy subjects were not demonstrated. This result may be due to the small sample sizes in this study, which may have resulted in the failure to identify a modest difference in *NRGN* expression in this complex disease. We determined that the major TG haplotypic and the TA haplotypic frequencies of rs12807809–rs12278912 were higher and lower, respectively, in patients with schizophrenia than in healthy controls. In addition to these findings, we found that *NRGN* gene expression of the high-risk TG haplotype was significantly lower than that of the protective TA haplotype in lymphoblasts derived from our Japanese case–control subjects as well as the JPT HapMap sample. The low LD patterns of rs12807809–rs12278912 were similar across populations. This region may be vulnerable to recombination. Combinations of the TG and TA of rs12807809–rs12278912 could play an important role in the pathogenesis of schizophrenia. In this study, gene expression data derived from lymphoblasts raised the possibility that the rs12807809–rs12278912 haplotype may be a functional variant of *NRGN*. Further biological studies of the function of rs12807809–rs12278912 are required to verify the expression results.

Smith et al. [2011] analyzed *NRGN* expression in several brain tissues derived from a dataset of at least 130 individuals of European ancestry. However, they showed that neither the genome-wide supported SNP nor any individually correlated SNPs were associated with *NRGN* expression. They did not examine any association between haplotype and *NRGN* expression. There are several challenges in investigating expression findings in the postmortem

TABLE III. The Association Between the rs12807809–rs12278912 Haplotype and mRNA Expression

Haplotypes	Frequency	Coefficient	SE	CI	*P*-value (*Z*-score)
Schizophrenia (*n* = 42)					
TG	0.69	0 (ref)	—	—	—
CG	0.16	−0.05	0.17	(−0.39–0.29)	0.76 (−0.30)
TA	0.07	0.37	0.25	(−0.12–0.86)	0.14 (1.49)
CA	0.08	−0.16	0.20	(−0.55–0.24)	0.43 (−0.78)
Healthy control (n = 44)					
TG	0.61	0 (ref)	—	—	—
CG	0.20	−0.09	0.16	(−0.39–0.22)	0.58 (−0.55)
TA	0.11	0.64	0.28	(0.09–1.18)	**0.021 (2.30)**
CA	0.09	−0.07	0.20	(−0.46–0.32)	0.73 (−0.34)
Total subjects (n = 86)					
TG	0.65	0 (ref)	—	—	—
CG	0.18	−0.06	0.11	(−0.28–0.15)	0.57 (−0.57)
TA	0.09	0.53	0.17	(0.19–0.87)	**0.002 (3.09)**
CA	0.08	−0.11	0.14	(−0.39–0.17)	0.45 (−0.75)

Joint Association Analysis (the reference haplotype is the most frequent haplotype).
For the joint haplotype test, several haplotypes were tested against the reference haplotype.
Significant *P* values are shown as bold-faced and underlined type.

brain: (1) the choice of an appropriate brain region for investigation; (2) the heterogeneity of cell types within brain tissue; (3) the reliance on relatively small samples; and (4) the impact of cause of death and/or postdeath handling of the tissues on gene expression [Marcotte et al., 2003]. Thus, the use of postmortem brain tissue is compounded by a range of confounding factors (age, race, gender, different microarray platforms, and analysis methods) and may be the cause of the relative lack of gene/transcript-level consistency among expression studies. To overcome some of these problems, several groups have considered the use of lymphoblasts rather than the postmortem brain [Matigian et al., 2008; Slonimsky et al., 2010; Yamamori et al., 2011; Yasuda et al., 2011]. Lymphoblasts are useful for schizophrenia researchers because blood-based tissue (lymphoblasts) can be obtained with ease from living subjects, which allows larger case–control studies with optimal matching of key variables (age, sex, and race). In addition, immortalized lymphoblasts in culture are considered an effective tool for studying cells in the absence of the effect of antipsychotic treatments and duration of illness, both of which could mask the genetic differences in RNA expression. Thus, lymphoblasts could be good tool to investigate the impact of a gene in the absence of the impact of any confounding factors. On the other hand, there were some demerits of using lymphoblasts. In immortalized lymphocytes, it might be difficult to observe the effects of genes on their neuron-specific functions, for example, the effects of genes on glutamate and dopamine release and on the formation of synaptic vesicles. When isolation and immortalization procedures of lymphocytes from blood were performed or immortalized lymphocytes were grown in culture media, a genetic mutation might be inserted into genomic DNA in the cultured lymphoblasts and alter DNA sequences. It remains still controversial whether immortalized lymphocytes are an appropriate alternative to neuronal tissue, because there was a little evidence of analysis using immortalized lymphocytes from patients with schizophrenia. In this study, the difference in the association of gene expression with genetic variants between previous study and present study could be explained by the difference in the gene expression profile between immortalized lymphoblast and postmortem brain tissue. Other possible factors contributing to differences in association between studies could be a difference in the SNPs and haplotypes investigated or ethnic differences between Japanese and Caucasian populations.

Smith et al. [2011] performed mutation searches of all four exons of *NRGN* gene in 14 Caucasian subjects with schizophrenia and of the cording exons of *NRGN* gene in 1,113 Bulgarians individuals, 699 of whom had schizophrenia. However, they did not find any novel common polymorphism in the region. Thus, we did not perform a systematic mutation search in this study because there has been no novel common genetic variant in the region. If we perform sequencing and find a novel rare polymorphism, we cannot analyze association between the rare polymorphism and gene expression for only a small number of individuals with rare variant. A genetic variant, particularly a SNP not listed in the HapMap database, that is likely to be more strongly associated with schizophrenia may exist in the rs12807809–rs12278912 haplotype region. Sequencing the entire gene in individuals with risk haplotype in comparison with the protective haplotype carriers with larger sample sizes could provide further information underlying the genomic mechanism for this risk haplotype.

There are several limitations to interpreting our results. Because a number of statistical analyses supported the association of the *NRGN* gene and schizophrenia, such as genotypic and allelic associations for five SNPs (total 5×2), haplotype analysis using a window fashion analysis (total 10) and expression analysis for three individual haplotypes (total 3×4), a correction for multiple testing should be considered. In this study, the overall number of genetic association tests was 32; however, not all tests were independent, and several hypotheses were included. Thus, Bonferroni correction, a method to correct for multiple independent tests for one hypothesis, might not be appropriate. The consensus how to correct such multiple testing has not been reached in this research field. Thus, we applied SNPSpD correction for genotypic and allelic association analysis, permutation method for haplotype analysis and Bonferroni correction for expression analysis (three tests). However, even though we applied these methods of correcting such multiple testing, they might cause false positive results. We did not control for geographical variation of control origin because there is little possibility for ethnic/genetic difference among four geographical regions for feature of homogeneous race in Japan [Yamaguchi-Kabata et al., 2008]. Our significant results may be derived from sample bias owing to population stratification and non-sex-matched samples. In the present study, our results support an association between the *NRGN* gene and schizophrenia. We suggest that the functional haplotype of the *NRGN* gene, which is associated with *NRGN* expression, could be related to the pathogenesis of schizophrenia.

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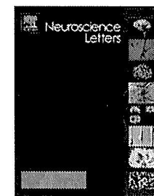

A promoter variant in the *chitinase 3-like 1* gene is associated with serum YKL-40 level and personality trait

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ABSTRACT

The *chitinase 3-like 1* (*CHI3L1*) gene, a cellular survival factor against several environmental and psychosocial stresses, has been sown to be more highly expressed in the hippocampus and prefrontal cortex of patients with schizophrenia than unaffected individuals. We recently reported a significant association between schizophrenia and SNP rs4950928, which is located in the promoter region of the *CHI3L1* gene, in a Japanese population. The G-allele at this SNP in the gene has been associated with higher transcriptional activity in a luciferase reporter assay and with higher mRNA levels in the peripheral blood cells of patients with schizophrenia. We investigated the impact of the *CHI3L1* polymorphism rs4950928 on serum YKL-40 levels, the protein product of *CHI3L1*. We found that individuals with the G-allele, who were more prevalent among patients with schizophrenia, had significantly higher serum YKL-40 levels ($p = 0.043$). Personality traits are considered to be an important aspect of schizophrenia primarily because they may influence symptoms and social functioning. Personality trait analyses using the temperament and character inventory (TCI) indicated that schizophrenic patients have a unique personality profile that appears to be present across cultures. We hypothesized that higher serum YKL-40 levels are associated with personality trait in patients with schizophrenia. Thus, we next examined the impact of the risk *CHI3L1* polymorphism on personality traits using the TCI. We found that individuals with the G-allele had significantly higher self-transcendence scores ($p = 0.0054$). These findings suggest possible associations between the SNP in the *CHI3L1* gene, the risk for schizophrenia, and higher serum YKL-40 levels and personality traits in a Japanese population.

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Schizophrenia is a common and complex psychiatric disease. Many genes have been implicated in the pathogenesis of schizophrenia [8,9,19,30,33], and the *chitinase 3-like 1* gene (*CHI3L1*) gene has been reported to be associated with the disease [34,35]. We have recently reported a significant association between schizophrenia and a SNP rs4950928 ($p = 0.009$) located in the promoter region of the *CHI3L1* gene (the most significant $p < 0.001$) in a Japanese population using the largest sample size to date (1463 cases and 1795 controls) [25]. Elevated expression of the *CHI3L1* gene has been indicated in the schizophrenic hippocampus and prefrontal cortex in independent postmortem studies [1,5]. The G-allele of the gene at rs4950928, which was found to be more prevalent in patients with schizophrenia, has been associated with higher transcriptional activity in a luciferase reporter assay and higher mRNA levels in peripheral blood cells in patients with schizophrenia [35]. *CHI3L1* gene acts as a cellular survival factor in responses to a variety of adverse environments, including various types of physiologic stress such as inflammation, hypoxia and nutrient deprivation. These stressors may induce high expression of *CHI3L1* [15,26]. The protein product of the *CHI3L1* gene was named YKL-40 [14]. YKL-40 is a secreted protein, produced by activated macrophages and neutrophils in different tissues characterized by inflammation and increased remodeling of the extracellular matrix [16,28,32]. YKL-40 initiates phosphoinositide-3 kinase (PI-3K) signaling cascades

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in fibroblasts [27]. The PI-3K pathway, in particular the phosphorylation of protein kinase B (AKT), is strongly associated with cell survival [2], which suggests a role for YKL-40 as an anti-apoptotic protein. The genetic variants of the *CHI3L1* gene and the higher serum YKL-40 levels are associated with several inflammatory diseases, such as sarcoidosis, asthma and inflammatory bowel diseases [13,17,18,24]. It has been hypothesized that YKL-40 plays a protective role in inflammatory processes in patients with schizophrenia and is highly expressed in patients with schizophrenia. In this study, we investigated whether the G-allele has an effect on YKL-40 levels in schizophrenic patients. To achieve this goal, we measured the serum YKL-40 levels of patients with schizophrenia and control subjects.

Personality traits are considered to be an important aspect of schizophrenia primarily because they may influence symptoms and social functioning [20,21]. The temperament and character inventory (TCI) is a well-established self-report questionnaire. It measures four temperament dimensions [novelty seeking (NS), harm avoidance (HA), reward dependence (RD) and persistence (PS)] and three character dimensions [self-directedness (SD), cooperativeness (CO) and self-transcendence (ST)] [6]. Personality trait analyses using the TCI have indicated that schizophrenic patients have a unique personality profile that appears to be present across cultures [higher scores of ST and HA and lower scores of NS, RD, SD and CO in schizophrenia] [3,4,7,11,29]. We hypothesized that higher serum YKL-40 levels would be associated with personality traits in patients with schizophrenia. Thus, we secondly examined the impact of the risk *CHI3L1* polymorphism on personality traits using the TCI.

For serum YKL-40 measurements, 20 patients with schizophrenia and 19 controls were enrolled. The subjects for personality trait analysis consisted of 99 patients with schizophrenia and 179 controls. All controls and 18 of 20 patients with schizophrenia enrolled for serum YKL-40 measurements were also enrolled for personality trait analysis. Cases were recruited at Osaka University hospitals. Each schizophrenic research subject had been diagnosed and assessed by at least two trained psychiatrists according to the Diagnostic and Statistical Manual of Mental Disorders, fourth edition (DSM-IV) criteria based on unstructured clinical interview. Symptoms of schizophrenia were assessed using the positive and negative syndrome scale (PANSS). Three of 20 patients with schizophrenia enrolled for serum YKL-40 measurements were not treated with anti psychotic drugs and 7 of 99 patients with schizophrenia enrolled for personality trait analysis were not treated with anti psychotic drugs. Cases of schizophrenia with the comorbidities of substance-related disorders or mental retardation were excluded. Controls were recruited through local advertisements. Psychiatrically, medically and neurologically healthy controls were evaluated using the DSM-IV structured clinical interview, non-patient version. Subjects were excluded if they had neurological or medical conditions that could potentially affect the central nervous system, such as atypical headache, head trauma with loss of consciousness, chronic lung disease, kidney disease, chronic hepatic disease, thyroid disease, active stage cancer, cerebrovascular disease, epilepsy or seizures. Written informed consent was obtained for all subjects after the procedures had been fully explained. This study was carried out in accordance with the World Medical Association's Declaration of Helsinki and approved by the Research Ethical Committee of Osaka University.

Serum YKL-40 was measured using an enzyme-linked immunosorbent assay kit (Metra YKL-40, Quidel Corporation, San Diego, CA, USA), in accordance with the manufacturer's instructions. All samples were run in duplicate and mean values were used for analysis. The average intra-assay coefficient of variation determined by triplicate of 10 CSF samples was 3.2%. To examine reproducibility, four CSF samples were analyzed in each of two experiments. After normalization, the average inter-assay coefficient of variation was 5.6%.

The TCI is administered through a self-report questionnaire based on 240 items requiring a true or false item response [6]. We only examined the main scores of the four temperaments (HA, NS, RD and PS) and three characters (SD, CO and ST) dimensions of the scale. The concepts of each dimension are as follows: NS is the activation of behavior in response to novelty and signals of reward or relief of punishment; HA is the inhibition of behavior in response to signals of punishment or non-reward; RD is the maintenance of behavior that was previously rewarded; PS is the perseveration with behavior despite frustration and fatigue; SD is the concept of the self as an autonomous individual; CO is the concept of the self as an integral part of humanity or society; and ST is the concept of the self as an integral part of the universe and its source [6].

Venous blood was collected from the subjects and genomic DNA was extracted from whole blood according to standard procedures. The timing of blood collection was not consistent among the samples. Genotyping of the SNP was carried out via TaqMan assays (Applied Biosystems, Foster City, CA, USA) as previously described [10,23]. The TaqMan probe and Universal PCR Master Mix were obtained from Applied Biosystems. The TaqMan probe ID for the SNP rs4950928 was C__27832042_10. Allelic-specific fluorescence was measured using an ABI PRISM 7900 Sequence Detector System (Applied Biosystems).

Statistical analyses were performed using SNPAlyze V5.1.1 Pro software (DYNACOM, Yokohama, Japan) and SPSS 16.0J software (SPSS Japan Inc., Tokyo, Japan). Differences in clinical characteristics between patients and controls or between genotype groups were analyzed using χ^2 tests for categorical variables and the Mann–Whitney *U*-test for continuous variables. Deviation from Hardy–Weinberg equilibrium (HWE) was tested separately in cases and controls. The analysis revealed age and gender differences in some dimensions. Therefore, the effect of the *CHI3L1* genotype and the effect of diagnosis on the serum YKL-40 levels were analyzed by a two-way analysis of covariance (ANCOVA), with age and gender as covariates. In previous personality traits analyses using the TCI, it has been suggested that possible confounding factors affect personality traits [22,31]. The number of years of education was lower in patients with schizophrenia than in healthy controls in a Japanese population [11]. Therefore, with age, gender and education years as covariates, the effect of *CHI3L1* genotype and the effect of diagnosis on personality traits were analyzed by a two-way ANCOVA. The significant level for statistical tests of genetic and personality association was set at $p < 0.05$.

We examined possible associations between the *CHI3L1* genotype at rs4950928 and serum YKL-40 levels in patients with schizophrenia and controls, because this variant was indicated to have a significant association with schizophrenia in a previous study. Supplementary Table 1 shows the characteristics of the subjects and the distribution of genotypes. There was no difference in demographic variables, age, gender, years of education, chlorpromazine equivalents of total antipsychotics (CPZeq) and positive and negative symptom scale (PANSS) scores between *CHI3L1* genotype groups. Given that there was only one CC homozygous individual in subjects for serum YKL-40 analysis, we removed the data of the subject with CC genotype and compared GG genotype with GC genotype. The effects of *CHI3L1* genotype and diagnosis on serum YKL-40 levels were shown in Table 1. Two-way ANCOVA revealed significant effects of genotype ($F = 4.46$, $p = 0.043$, $\eta^2 = 0.122$). No effect of diagnosis ($p > 0.70$) or genotype–diagnosis interaction was found ($p > 0.80$). Individuals homozygous for the G-allele, which was more common in the patient group, showed higher serum YKL-40 levels than the C-carriers (Fig. 1). There was no genotype effect when we separately analyzed the effect of genotype on YKL-40 in patients ($p > 0.30$) and controls ($p > 0.10$).

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Table 1
Effects of *CHI3L1* genotype and diagnosis on serumYKL-40.

Variables	Schizophrenia (n = 19)		Control (n = 19)		ANCOVA p-values (F-values)		
	G/G	G/C	G/G	G/C	Diagnosis effect	Genotype effect	Interaction
Serum YKL-40 (ng/ml)	109.8 ± 63.5	75.3 ± 15.6	101.7 ± 37.3	75.0 ± 24.0	0.696 (0.156)	*0.043 (4.46)*	0.785 (0.076)

Means ± SD are shown. ANCOVA, two-way analysis of covariance. Significant p-values are italicized.

Table 2
Effects of *CHI3L1* genotype, diagnosis and their interaction on personality traits using TCI.

	Schizophrenia		Control		ANCOVA p-values (F-values)		
	G/G(N = 70)	G/C, C/C(N = 29)	G/G(N = 118)	G/C, C/C(N = 61)	Diagnosis effect	Genotype effect	Interaction
NS	17.6 ± 4.8	18.4 ± 4.7	21.4 ± 4.4	20.5 ± 4.7	*2.21×10^{-6} (23.40)*	0.82 (0.05)	0.46 (0.54)
HA	22.6 ± 7.4	23.7 ± 6.3	16.3 ± 5.5	17.2 ± 5.8	*2.93×10^{-11} (48.13)*	0.24 (1.42)	0.89 (0.02)
RD	14.3 ± 3.5	14.2 ± 3.7	16.4 ± 2.9	15.9 ± 3.5	*8.08×10^{-4} (11.48)*	0.65 (0.21)	0.49 (0.48)
PS	4.4 ± 1.6	4.2 ± 1.7	4.6 ± 1.9	4.4 ± 1.7	0.96 (<0.01)	0.36 (0.83)	0.93 (0.01)
SD	24.3 ± 7.1	23.3 ± 8.8	29.4 ± 5.7	29.6 ± 5.7	*1.40×10^{-7} (29.24)*	0.72 (0.13)	0.96 (<0.01)
CO	27.2 ± 5.7	26.8 ± 6.3	29.3 ± 4.1	29.2 ± 5.2	*0.0090 (6.92)*	0.88 (0.02)	0.70 (0.15)
ST	13.9 ± 7.0	11.2 ± 6.1	9.7 ± 5.2	8.2 ± 4.2	*5.95×10^{-5} (16.64)*	*0.0054 (7.86)*	0.35 (0.89)

TCI, temperament and character inventory; NS, novelty seeking; HA, harm avoidance; RD, reward dependence; PS, persistence; SD, self directedness; CO, cooperativeness; ST, self transcendence. Means ± SD are shown. The effects of CHI3L1 genotype, diagnosis and their interaction on the personality trait were analyzed by a two-way analysis of covariance (ANCOVA) with age, gender and education years as covariates. Significant p-values are italicized.

We next examined possible associations between the *CHI3L1* genotype at rs4950928 and personality traits in patients with schizophrenia and in controls. There was no difference in demographic variables, age, gender, years of education, CPZeq and PANSS scores between the *CHI3L1* genotype groups (Supplementary Table 2). Given that there were few homozygous CC individuals, we divided the participants into two groups (individuals with GG genotype and C-carriers). The effects of *CHI3L1* genotype and diagnosis on personality traits as measured by TCI are shown in Table 2. Two-way ANCOVA revealed significant effects of diagnosis (NS: $F = 23.40$, $p < 0.001$; HA: $F = 48.13$, $p < 0.001$; RD: $F = 11.48$, $p < 0.001$; SD: $F = 29.24$, $p < 0.001$; CO: $F = 6.92$, $p < 0.001$ and ST: $F = 16.64$, $p < 0.001$) and genotype (ST: $F = 7.86$, $p = 0.0054$, $\eta^2 = 0.03$). No genotype–diagnosis interaction was found ($p > 0.20$). Individuals homozygous for the G-allele had higher ST scores than the C-carriers (Fig. 2).

To our knowledge, this is the first report showing an association between the G-allele at rs4950928 and higher serum YKL-40 levels in both patients with schizophrenia and controls. The G-allele may be related to the part of pathophysiology of schizophrenia through its effect on serum YKL-40 levels. Higher serum YKL-40 levels may be the response to the environmental and psychological stresses that have been shown to be sensitive in schizophrenia [12].

We first performed the association study to assess *CHI3L1* genotype and personality traits using 99 patients and 179 controls, which is the largest sample size to date for an association study examining a risk genotype and the TCI. Similar to the previous studies, we found significantly lower scores for NS, RD and SD and higher scores on HA and ST in patients than in controls (diagnosis effect). Moreover, higher ST scores were also revealed in individuals with the risk GG genotype at rs4950928 compared to the C-carriers (genotype effect). For personality traits, however, no genotype–diagnosis interaction was detected. ST is an important dimension for schizophrenia, as higher ST scores have been shown in unaffected relatives of schizophrenic patients [3]. Therefore, this dimension might be an intermediated-phenotype for schizophrenia.

This study has several limitations. The sample size was relatively small for both the serum YKL-40 and TCI analyses. In the previous multi-center case–control study which showed the significant association between schizophrenia and SNP rs4950928 in

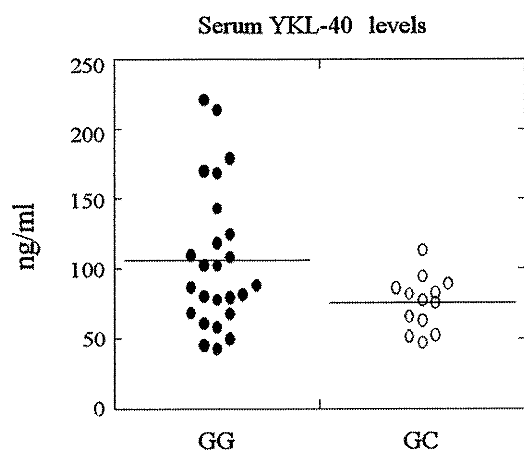

Fig. 1. Effect of the risk *CHI3L1* genotype at SNP rs4950928 on serum YKL-40 levels. *X* axis represents *CHI3L1* genotypes at SNP rs4950928. *Y* axis represents serum YKL-40 levels.

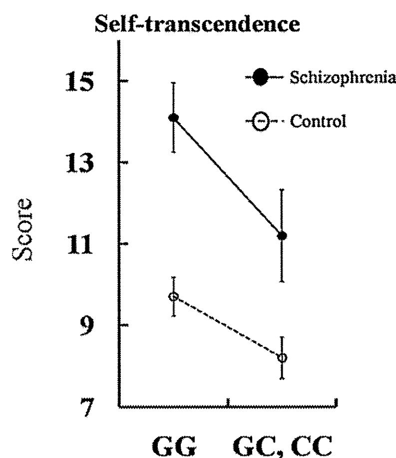

Fig. 2. Effect of the risk *CHI3L1* genotype at SNP rs4950928 and effect of diagnosis on personality trait (self-transcendence) using TCI. *X* axis represents *CHI3L1* genotypes at SNP rs4950928. *Y* axis represents self-transcendence scores. Closed circles represent subjects with schizophrenia. Open circles represent healthy controls. Bars represent the standard error.

a Japanese population [25], 1463 cases and 1795 controls were enrolled, however, serum samples and personality traits data were available only in limited cases and controls from our laboratory. For TCI analysis, we had enough power of 0.802 (sample size: 278, effect size: $\eta^2 = 0.03$, statistical significance = 0.05). For serum YKL-40 analysis, we did not have enough power: 0.599 < 0.8 (sample size: 38, effect size: $\eta^2 = 0.122$, statistical significance = 0.05), however, we fortunately found the significant association. Because of the small sample size, only one CC homozygous individual was included in serum YKL-40 analysis and we removed the data of the subject with CC genotype and compared GG genotype with GC genotype. A much larger sample size would be needed to definitively test the associations between the *CHI3L1* genotype and YKL-40 and TCI in schizophrenia. Circadian rhythms and medications may affect the serum YKL-40 levels, however, the timing of blood collection was not consistent among the samples. In addition, personality traits were assessed in patients after the onset of symptoms. A careful interpretation of our results is called for because we did not consider whether the findings reflect pre-clinical personality traits versus pre- or post-therapeutic personality traits. Further replication studies in other ethnic populations and an association study between the risk *CHI3L1* genotype and serum YKL-40 levels and personality trait in patients with schizophrenia are required to establish a definitive relationship between *CHI3L1* and schizophrenia.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.neulet.2012.02.039.

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Impact of the Genome Wide Supported *NRGN* Gene on Anterior Cingulate Morphology in Schizophrenia

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Abstract

Background: The rs12807809 single-nucleotide polymorphism in *NRGN* is a genetic risk variant with genome-wide significance for schizophrenia. The frequency of the T allele of rs12807809 is higher in individuals with schizophrenia than in those without the disorder. Reduced immunoreactivity of *NRGN*, which is expressed exclusively in the brain, has been observed in Brodmann areas (BA) 9 and 32 of the prefrontal cortex in postmortem brains from patients with schizophrenia compared with those in controls.

Methods: Genotype effects of rs12807809 were investigated on gray matter (GM) and white matter (WM) volumes using magnetic resonance imaging (MRI) with a voxel-based morphometry (VBM) technique in a sample of 99 Japanese patients with schizophrenia and 263 healthy controls.

Results: Although significant genotype-diagnosis interaction either on GM or WM volume was not observed, there was a trend of genotype-diagnosis interaction on GM volume in the left anterior cingulate cortex (ACC). Thus, the effects of *NRGN* genotype on GM volume of patients with schizophrenia and healthy controls were separately investigated. In patients with schizophrenia, carriers of the risk T allele had a smaller GM volume in the left ACC (BA32) than did carriers of the non-risk C allele. Significant genotype effect on other regions of the GM or WM was not observed for either the patients or controls.

Conclusions: Our findings suggest that the genome-wide associated genetic risk variant in the *NRGN* gene may be related to a small GM volume in the ACC in the left hemisphere in patients with schizophrenia.

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Introduction

Schizophrenia is a common and complex psychiatric disorder that has a strong genetic component; the estimated heritability is 81% [1]. Many genes have been implicated in the pathogenesis of schizophrenia [2].

A genome-wide association study (GWAS) of single-nucleotide polymorphisms (SNPs) conducted by accessing thousands of DNA samples from patients and controls can be a powerful tool for identifying common risk factors for such a complex disease. Stefansson et al. examined a combined sample of 12,945 patients with schizophrenia and 34,591 controls from three large GWASs (the SGENE-plus, the International Schizophrenia Consortium and the Molecular Genetics of Schizophrenia) and a follow-up with 4,999 patients and 15,555 controls from four additional sample sets from various areas of Europe (including the Netherlands, Denmark, Germany, Hungary, Norway, Russia, Sweden, Finland and Spain) [3]. The researchers identified several

significant association signals. Seven markers gave *p* values smaller than the genome-wide significance threshold of approximately 1.6×10^{-7} in the combined samples. Five of these markers—rs6913660, rs13219354, rs6932590, rs13211507 and rs3131296—span the major histocompatibility complex (MHC) region on chromosome 6p21.3–22.1; one marker, rs12807809, is located 3,457 bases upstream from the neurogranin (*NRGN*) gene on 11q24.2; one additional marker, rs9960767, is located in intron four of the transcription factor 4 (*TCF4*) gene on 18q21.2. Of these seven SNPs, four SNPs, rs6913660, rs13219354, rs13211507 and rs9960767, were not polymorphic in samples from the HapMap Japanese in Tokyo (JPT) project. Minor allele frequencies (MAF) of two SNPs, rs6932590 and rs3131296, were under 5%. Because only one marker, rs12807809 in *NRGN*, was a common SNP in HapMap JPT samples (MAF>5%), we focused on this SNP in the present study.

NRGN is the human homolog of the neuron-specific rat gene RC3/neurogranin. *NRGN* encodes a postsynaptic protein kinase substrate that binds to calmodulin (CaM) in the absence of calcium [4]. The *NRGN* gene spans 7.3 kb of genomic DNA and contains four exons that transcribe a protein of 78 amino acids [5]. Exons 1 and 2 encode the protein, and exons 3 and 4 contain untranslated sequences. NRGN plays an important role in the Ca^{2+}–CaM signaling pathway [6]. A Ca^{2+} influx-induced oxidation of NRGN leads to postsynaptic activation of CaM-dependent protein kinase II (CaMKII) by CaM, which is associated with strengthened *N*-methyl-D-aspartate (NMDA) receptor signaling [7]. Altered NRGN activity may therefore mediate the effects of the NMDA hypofunction implicated in the pathophysiology of schizophrenia.

Many attempts have been made to minimize clinical and genetic heterogeneity in studies of schizophrenia. One strategy for gene discovery uses neurobiological quantitative traits (QT) as intermediate phenotypes rather than the diagnosis of schizophrenia [8,9]. This strategy has the potential to reduce clinical and genetic heterogeneity by examining intermediate phenotypes that reflect underlying genetic vulnerability better than diagnostic categorization [10]. Structural brain phenotypes are QT that show considerable variation in human populations [11]. A voxel-wise meta-analysis of gray matter (GM) alterations in patients with schizophrenia indicated that they had a reduced GM density in the bilateral insular cortex, anterior cingulate, left parahippocampal gyrus, left middle frontal gyrus, postcentral gyrus, and thalamus and had an increased GM density in the striatal regions relative to the control subjects [12]. A voxel-wise meta-analysis of white matter (WM) alterations in patients with schizophrenia indicated that these patients had a decreased WM volume in the frontal regions and internal capsule relative to control subjects [13]. Heritability estimates indicate a moderate (40–70%) to high (70–95%) genetic influence on brain structure volumes in the frontal and temporal brain regions, such as the middle frontal and the anterior cingulate cortices [11,14]. Some studies have shown that abnormalities in brain structure are intermediate phenotypes that bridge the gap between the genotype and diagnostic categorization [10,15,16]. Our research group has a long-standing interest in the effects of genetic variants on brain structure (i.e., *COMT, DISC1, PACAP, BDNF, APOE* and *AKT1*) [17,18, 19,20,21,22] and on prefrontal activity as measured by near-infrared spectroscopy (NIRS) (*TBP* and *SIGMAR1*) in psychiatric disorders [23,24]. *NRGN* is expressed exclusively in the brain, especially in the dendritic spines. Reduced NRGN immunoreactivity has been observed in prefrontal areas 9 and 32 of post-mortem schizophrenic brains [25]. To date, no study has investigated the effects of the *NRGN* polymorphism and the genotype-diagnosis interaction on brain morphology at the whole brain level. In this study, we examined the impacts of the *NRGN* polymorphism and the genotype-diagnosis interaction on GM volumes and WM volumes in patients with schizophrenia and in healthy volunteers.

Materials and Methods

Ethics statement

Written informed consent was obtained from all subjects after the procedures had been fully explained. This study was carried out in accordance with the World Medical Association's Declaration of Helsinki and approved by the Research Ethical Committee of Osaka University.

Subjects

Voxel-based morphometry (VBM) analyses were conducted on 99 patients with schizophrenia [52.5% males (52 males and 47 females); mean age ± SD, 38.4±12.9 years] and 263 healthy controls [44.5% males (117 males and 146 females); mean age ± SD, 36.7±11.6 years]. All subjects were biologically unrelated within the second-degree of relationship and of Japanese descent [23,26]. The subjects were excluded if they had neurological or medical conditions that could potentially affect the central nervous system, such as atypical headache, head trauma with loss of consciousness, chronic lung disease, kidney disease, chronic hepatic disease, thyroid disease, active cancer, cerebrovascular disease, epilepsy, seizures, substance-related disorders or mental retardation. Cases were recruited from the university hospital. Each patient with schizophrenia had been diagnosed by at least two trained psychiatrists according to the criteria of the *Diagnostic and Statistical Manual of Mental Disorders, Fourth Edition* (DSM-IV) based on the Structured Clinical Interview for DSM-IV (SCID). Controls were recruited through local advertisements at Osaka University. Psychiatrically, medically and neurologically healthy controls were evaluated using the non-patient version of the SCID to exclude individuals who had current or past contact with psychiatric services or who had received psychiatric medication. Current symptoms of schizophrenia were evaluated using the positive and negative syndrome scale (PANSS) [27]. Mean age, sex ratio and handedness did not differ significantly between cases and controls ($p>0.17$), while the years of education, estimated premorbid intelligence quotient (IQ) and GM volumes were significantly lower in the patients with schizophrenia than in the controls ($p<0.001$) (Table S1). When the genotype groups were compared, we found no differences in the demographic variables, except for years of education and duration of illness in patients with schizophrenia (Table S1).

SNP selection and SNP genotyping

We selected rs12807809 in the *NRGN* gene as described in the introduction. This polymorphism is reported as T/C and was previously described in the GWAS [3]. Venous blood was collected from the subjects, and genomic DNA was extracted from whole blood according to standard procedures. The SNP was genotyped using the TaqMan 5′-exonuclease allelic discrimination assay (Assay ID: C__32029000_20, Applied Biosystems, Foster City, California, USA) as previously described [18,19]. Detailed information on the PCR conditions is available upon request. No deviation from Hardy-Weinberg equilibrium (HWE) in the examined SNP was detected in the patients or in the controls ($p>0.05$).

Magnetic resonance imaging procedure

All magnetic resonance (MR) studies were performed on a 1.5T GE Sigma EXCITE system. A three-dimensional volumetric acquisition of a T1-weighted gradient echo sequence produced a

gapless series of 124 sagittal sections using a spoiled gradient recalled acquisition in the steady state (SPGR) sequence (TE/TR, 4.2/12.6 ms; flip angle, 15°; acquisition matrix, 256×256; 1NEX, FOV, 24×24 cm; slice thickness, 1.4 mm). MR images were processed using optimized VBM in Statistical Parametric Mapping 5 (SPM5) running on MATLAB R2010b (MathWorks, Natick, MA) according to the VBM5.1-Manual (http://dbm.neuro.uni-jena.de/vbm/vbm5-for-spm5/manual/) and as previously described [28,29]. We screened all scans and found no gross abnormalities, such as infarcts, hemorrhages or brain tumors, in any of the subjects. Each image was visually examined to eliminate images with motion or metal artifacts, and then the anterior commissure-posterior commissure line was adjusted. The normalized segmented images were modulated by multiplication with Jacobian determinants of the spatial normalization function to encode the deformation field for each subject as tissue volume changes in the normal space. Finally, images were smoothed with a 12-mm full-width, half-maximum isotropic Gaussian kernel.

Statistical analyses were performed with SPM8 software (http://www.fil.ion.ucl.ac.uk/spm/software/spm8/). First, we performed whole brain searches to explore the effects of the *NRGN* genotype and the genotype-diagnosis interaction on GM or WM volume in total subjects. Second, we performed separate whole brain searches to explore the effect of the *NRGN* genotype on GM or WM volume in patients with schizophrenia and in controls. The genotype effect on GM or WM volume was assessed statistically using a multiple regression model in SPM8. We contrasted GM or WM volume between the genotype groups (coded as the number of rs12807809 risk T alleles: 0, 1, or 2); GM or WM volumes were correlated with the number of risk T alleles, either positively (CC<CT<TT) or negatively (TT<CT<CC). The genotype-diagnosis interaction on GM or WM volumes was assessed full factorial model with diagnosis as a factor and genotype status as a covariate interacted with the diagnosis in SPM8. Age, sex and years of education were included as covariates of no interest into all analyses to control for confounding variables. Non-sphericity was estimated. These analyses yielded statistical parametric maps {SPM (t)} based on a voxel-level height threshold of $p<0.001$ (uncorrected for multiple comparisons). Clusters of more than 100 contiguous voxels were considered in the analyses. Family-wise error (*FWE*) correction was applied for multiple testing to avoid type I errors. The significance level was set at $p<0.05$ (*FWE* corrected). Anatomic localization was performed according to both MNI coordinates and Talairach coordinates, which were obtained from M. Brett's transformations (http://www.mrccbu.cam.ac.uk/Imaging/Common/mnispace.shtml) and presented as Talairach coordinates.

Statistical analyses

The presence of Hardy-Weinberg equilibrium was examined by the χ^2 test for goodness-of-fit using SNPAlyze V5.1.1 Pro software (DYNACOM, Yokohama, Japan). Statistical analyses of demographic variables were performed using PASW Statistics 18.0 software (SPSS Japan Inc., Tokyo, Japan). Differences in clinical characteristics between patients and controls or between genotypes were analyzed using χ^2 tests for categorical variables and the Mann-Whitney *U*-test or Kruskal-Wallis test for continuous variables. The significance level for all statistical tests was set at two-tailed $p<0.05$.

Results

Effects of the genotype and diagnosis-genotype interaction on GM or WM regions in total subjects

First, we investigated the effects of genotype and diagnosis-genotype interaction on GM or WM volumes in the whole brain analyses of total subjects. We found significant effects of the risk T allele on decreased GM volume in the right fusiform gyrus (uncorrected $p<0.001$, Table 1 and blue regions in Figure 1), and on increased WM volume in the inferior parietal lobule among total subjects (uncorrected $p<0.001$, Table 1). We also found significant genotype-diagnosis interaction on GM volume in the left anterior cingulate gyrus and the bilateral precuneus (uncorrected $p<0.001$, Table 1 and red regions in Figure 1). However, the effects of genotype and genotype-diagnosis interaction on these GM or WM regions did not survive after the *FWE*-correction for multiple tests (*FWE*-corrected $p>0.05$). There was no significant effect of the risk T allele on increased GM volumes, the risk T allele on decreased WM volumes, or genotype-diagnosis interaction on WM volume among total subjects (uncorrected $p>0.001$).

Effect of the risk T allele on decreased GM regions (TT<CT<CC)

Second, we separately investigated the effects of genotype on GM or WM volumes in the whole brain analyses of patients with schizophrenia and healthy controls. We found significant effects of the *NRGN* genotype on GM volume in the left anterior cingulate gyrus, the bilateral middle temporal gyrus and the left inferior frontal gyrus among the patients with schizophrenia (uncorrected $p<0.001$, Table 2 and red regions in Figure S1). We found significant effect of the *NRGN* genotype on GM volume in the right fusiform gyrus among the healthy controls (uncorrected $p<0.001$, Table 2 and blue regions in Figure S1). The genotype effect on the left anterior cingulate gyrus (BA32) in the patients with schizophrenia remained significant even after the *FWE*-correction for multiple tests at the whole brain level ($T_{94}=5.63$, *FWE*-corrected $p=0.0042$, Table 2); genotype effects on other regions did not survive the *FWE*-correction (*FWE*-corrected $p>0.05$). In patients with schizophrenia, the risk T carriers had a smaller GM volume in the left anterior cingulate gyrus than did the non-risk C carriers (Figure 2).

Researchers have suggested that the volume reduction of the anterior cingulate cortex (ACC) is associated with the duration of the illness (the length of time the patient has had schizophrenia) [30]. In our samples, the duration of illness differed significantly among the genotype groups in patients with schizophrenia (Table S1). Thus, we corrected for the duration of illness. The genotype effect on the left anterior cingulate gyrus remained significant even after controlling for the duration of illness ($T_{93}=5.86$, *FWE*-corrected $p=0.0017$).

Effect of the risk T allele on increased GM regions (CC<CT<TT)

We found significant effects of the *NRGN* genotype on GM volume in the bilateral precuneus among the patients with schizophrenia (uncorrected $p<0.001$, Table 2 and red region in Figure S2); however, the genotype effects on these regions did not survive after the *FWE*-correction for multiple tests (*FWE*-corrected $p>0.05$). There was no significant effect of the *NRGN* genotype on GM volume among the healthy controls (uncorrected $p>0.001$).

Effects of the risk T allele on WM regions

We found no significant effect of the risk T allele on any decreased WM regions (TT<CT<CC) for either the patients or controls (uncorrected $p<0.001$). On the other hand, we found significant effects of the risk T allele on increased WM region (CC<CT<TT) in the bilateral insula and middle frontal gyrus

Table 1. Effects of *NRGN* genotype and genotype-diagnosis interaction on GM and WM volumes in total subjects.

						p values		Talairach coordinates		
	Brain regions	**R/L**	**BA**	**CS**	*T*	**Uncorrected**	*FWE*	*x*	*y*	*z*
GM	***NRGN* genotipe-diagnosis interaction**									
	Limbic Lobe									
	Anterior Cingulate	L	32	219	4.17	<0.001	0.33	−12	40	−10
	Occipital Lobe									
	Precuneus	R	31	118	3.63	<0.001	0.90	15	−64	20
	Precuneus	L	31	165	3.54	<0.001	0.95	−7	−72	25
GM	**Total subjects; TT<CT<CC (higher risk<lower risk)**									
	Temporal Lobe									
	Fusiform Gyrus	R	20	290	4.28	<0.001	0.25	45	−30	−23
GM	**Total subjects; TT>CT>CC (higher risk>lower risk)**									
	no suprathreshold clusters									
WM	***NRGN* genotipe-diagnosis interaction**									
	no suprathreshold clusters									
WM	**Total subjects; TT<CT<CC (higher risk<lower risk)**									
	no suprathreshold clusters									
WM	**Total subjects; TT>CT>CC (higher risk>lower risk)**									
	Parietal Lobe									
	Inferior Parietal Lobule	R		616	3.72	<0.001	0.34	44	−41	25

GM: gray matter, WM: white matter, R: right, L: left, BA: Brodmann area, CS: Cluster size, FWE: family-wise error.
doi:10.1371/journal.pone.0029780.t001

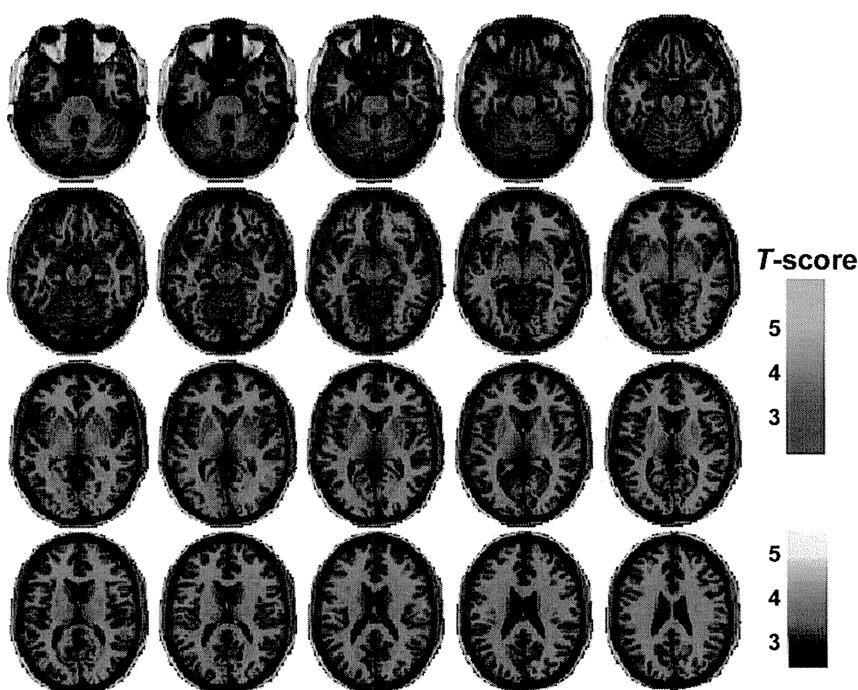

Figure 1. Effects of the risk-T-allele on decreased GM regions and diagnosis-*NRGN* genotype interaction on GM regions. Effects of the risk T allele on decreased GM regions (TT<CT<CC) in total subjects were shown by whinter colormap (blue areas). Diagnosis-*NRGN* genotype interaction on GM regions was shown by hot colormap (red areas). There was no significant effect of the risk T allele on increased GM regions (CC<CT<TT) among the total subjects. Each colormap shows *t* values corresponding to the color in the figure.
doi:10.1371/journal.pone.0029780.g001

Table 2. Effects of *NRGN* genotype on GM volumes in patients with schizophrenia and in healthy controls.

Brain regions	R/L	BA	CS	*T*	*p* values Uncorrected	*FWE*	Talairach coordinates *x*	*y*	*z*
SZ; TT<CT<CC (higher risk<lower risk)									
Limbic Lobe									
Anterior Cingulate	L	32	525	5.63	<0.001	**0.0042**	−12	42	−9
Temporal Lobe									
Middle Temporal Gyrus	L	21	143	3.87	<0.001	0.80	−66	−19	−5
Middle Temporal Gyrus	R	21	106	3.69	<0.001	0.93	59	−24	−6
Frontal Lobe									
Inferior Frontal Gyrus	L	10	102	3.88	<0.001	0.80	−36	45	4
HC; TT<CT<CC (higher risk<lower risk)									
Temporal Lobe									
Fusiform Gyrus	R	20	334	4.4	<0.001	0.19	45	−31	−23
SZ; TT>CT>CC (higher risk>lower risk)									
Parietal Lobe									
Precuneus	L	7	182	4	<0.001	0.68	−15	−64	38
Occipital Lobe									
Precuneus	R	31	143	3.81	<0.001	0.86	15	−64	19
HC; TT>CT>CC (higher risk>lower risk)									
no suprathreshold clusters									

GM: gray matter, R: right, L: left, BA: Brodmann area, CS: Cluster size, FWE: family-wise error, SZ: patients with schizophrenia, HC: healthy controls. Significant results [$p<0.05$ (*FWE* corrected)] are shown as bold face and underline.
doi:10.1371/journal.pone.0029780.t002

among the patients with schizophrenia (uncorrected $p<0.001$, Table S2 and red regions in Figure S3). However, the genotype effects on these regions did not survive after the *FWE*-correction (*FWE*-corrected $p>0.05$). There was no significant genotype effect on any increased WM region for the controls (uncorrected $p<0.001$). These findings suggest that *NRGN* may not play a major role in the morphology of WM.

Discussion

This is the first study to identify brain morphology associated with genome-wide significant risk variants in *NRGN* for schizophrenia at the whole brain level. Genotype-diagnosis interaction on GM volume in the left ACC was found, even though the effect did not survive after the *FWE*-correction. When we separately

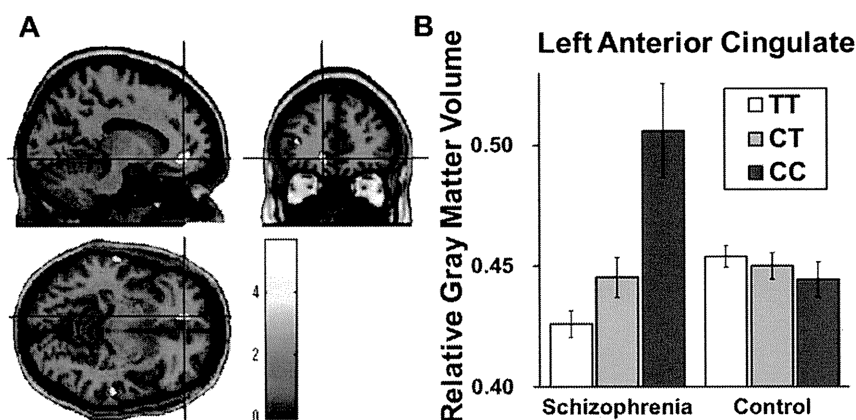

Figure 2. Impact of the *NRGN* genotype on GM volume of left anterior cingulate gyrus in schizophrenia. (**A**) Anatomical localizations are displayed on coronal, sagittal, and axial sections of a normal MRI spatially normalized into the Montreal Neurological Institute template (uncorrected $p<0.001$, cluster size>100). A significant cluster of the genotype effect was in the left anterior cingulate gyrus in the patients with schizophrenia, after controlling for differences in the duration of illness among genotypes. The region is shown as cross-hairline. The color bars show *t* values corresponding to the color in the figure. (**B**) Each column shows relative gray matter volumes extracted from the left anterior cingulate gyrus (Talairach coordinates; −12, 42, −9). We extracted a sphere with a 10 mm volume-of-interest (VOI) radius from the significant region to compare the effects of the genotype in both the patients with schizophrenia and healthy subjects. Error bars represent the standard error.
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investigated the effects of the interaction on GM volume of patients with schizophrenia and healthy controls, carrying the risk T allele of rs12807809 was associated with reduced GM volume in the left ACC in patients with schizophrenia. The genotype effect survived a correction for multiple comparisons at the whole brain level. This finding applies to the patients with schizophrenia but not to the healthy controls, and it is present even after controlling for differences in the duration of illness among genotypes. Significant difference on WM volume between genotypes was not observed for any region in patients or controls.

The ACC is a functionally heterogeneous region involved in diverse cognitive processes [30]. The functional diversity of the ACC encompasses executive, attention, social cognitive, affective and skeleton- and viscera-motor functions. Most MRI studies suggest that patients with schizophrenia show reduced GM in the ACC [30]. These reductions extend across the dorsal and rostral divisions of the limbic and paralimbic regions of the ACC. Some studies suggest that relatives of schizophrenia patients also show bilateral reductions in GM volume or thickness in the ACC [31,32]. Post-mortem findings indicate that these imaging-related changes are accompanied by reductions in neuronal, synaptic, and dendritic density as well as increased afferent input [30]. These findings suggest that the GM differences observed with MRI arise from alterations in both neuronal and non-neuronal tissue compartments.

The GM reductions in the ACC precede the onset of psychosis in some categories of high-risk individuals. Cross-sectional and longitudinal studies suggest that the earliest ACC changes in schizophrenia appear in the rostral paralimbic regions of the ACC prior to the onset of psychosis, extend across the paralimbic regions of the ACC during the transition to a first episode psychosis, and spread to engulf the limbic regions of the ACC with continued illness [30]. The regions of the genotype effect in the present study were the paralimbic regions of the ACC. A mean duration of illness in patients included in this study was 13.0 ± 10.4 years; these patients are considered to have established schizophrenia. As the duration of illness has been related to the degree of reduction of the ACC and because it significantly differed among the genotype groups in our subjects, we ascertained whether the genotype effect in the ACC is affected by variation in the duration of illness. However, the genotype effect in the left ACC was robust even after controlling for the duration of illness. These findings suggest that part of the paralimbic regions of the ACC may be attributed to the effects of the genome-wide supported variant of *NRGN* in patients with schizophrenia, regardless of the duration of illness.

NRGN is especially enriched in CA1 pyramidal neurons in the hippocampus [33]. *NRGN* produced severe deficits in hippocampus-dependent tasks in knock-out mice [34,35]. This evidence suggests that *NRGN* may be important in neurocognitive tasks such as learning and memory and in the morphology and function of the hippocampus. Based on this hypothesis, Donohoe et al. tested the relationship between schizophrenia associated with the *NRGN* variant rs12807809 and cognition in Irish and German case-control samples [36]. They did not find a significant association between the *NRGN* variant and cognition in the samples. Pohlack et al. found that homozygous T carriers had decreased activation of the left hippocampus during contextual fear conditioning but did not find the same result in the hippocampal structure of Caucasian healthy volunteers [37]. We did not find a significant association between the *NRGN* variant and hippocampal volume, consistent with recent study using the ROI approach [37]. These findings suggest that *NRGN* may play an important role in hippocampal activity but not play a major role in the neurocognition of learning and memory or in the morphology of the hippocampus.

There were several limitations to this study. A false-positive association could not be excluded from our study despite the precautions for ethnic matching and corrections for multiple testing. It is necessary to conduct further investigations to confirm our findings in other samples with much larger sample sizes and/or with different ethnicities and/or in relatives with schizophrenia. A false-negative association could not be excluded in our study because we applied a strict correction for multiple comparisons at the whole brain level (*FWE*-corrected $p<0.05$). The regions shown in the Supporting Information (uncorrected $p<0.001$) might be helpful in further studies. It is still unclear whether this genetic variant of the *NRGN* gene is associated with the expression, transcription, splicing or translation of the gene. The lack of a clear association makes it difficult to determine whether our results are directly linked to the *NRGN* polymorphism rs12807809, to other polymorphisms in linkage disequilibrium with this variant, or to interaction between this genetic variant of the *NRGN* and other polymorphism. As with other risk variants for schizophrenia, clarifying the biological role of this variant through *in vitro* and *in vivo* studies is important to improve the understanding of the pathophysiology of schizophrenia. In addition, an extensive search for other functional variants at this locus is needed to determine whether rs12807809 is the most strongly associated variant for schizophrenia in this gene.

In conclusion, we found that a genome-wide supported variant of *NRGN* may be associated with brain morphological vulnerability of the left ACC in patients with schizophrenia. Abnormalities in ACC may partly explain the disturbances in cognitive and emotional integration in patients with schizophrenia. Further research will be required to clarify the function of the risk *NRGN* variant on the pathophysiology of schizophrenia.

Supporting Information

Figure S1 Effect of risk-T-allele on decreased GM regions in patients with schizophrenia and in healthy controls. Effect of the risk T allele on decreased GM regions (TT<CT<CC) in the patients with schizophrenia was shown by hot colormap (red areas), while effect of the T allele on decreased GM regions in the healthy controls was shown by winter colormap (blue areas).
(TIF)

Figure S2 Effect of the risk-T-allele on increased GM regions in the patients with schizophrenia. Effect of the risk T allele on increased GM regions (CC<CT<TT) in the patients with schizophrenia was shown by hot colormap (red areas). There was no significant effect of the *NRGN* genotype on GM volume among the healthy controls.
(TIF)

Figure S3 Effect of the risk-T-allele on increased WM regions in the patients with schizophrenia. Effect of the risk T allele on increased WM regions (CC<CT<TT) in the patients with schizophrenia was shown by hot colormap (red areas). There was no significant effect of the *NRGN* genotype on WM volume among the healthy controls.
(TIF)

Table S1 Demographic information for patients with schizophrenia and healthy controls included in the VBM analysis.
(DOC)

Table S2 Effects of the *NRGN* genotype on WM volumes in patients with schizophrenia and healthy controls.
(DOC)

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Author Contributions

Conceived and designed the experiments: KO RH. Performed the experiments: HY SU TOakada KN TOhnishi. Analyzed the data: KO RH KN TOhnishi HY SU TOakada. Contributed reagents/materials/analysis tools: YY MF MI HK MT. Wrote the paper: KO RH YY MF MI HK MT.

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