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The impact of the genome-wide supported variant in the cyclin M2 gene on gray matter morphology in schizophrenia

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Abstract

Background: Genome-wide significant associations of schizophrenia with eight SNPs in the *CNNM2*, *MIR137*, *PCGEM1*, *TRIM26*, *CSMD1*, *MMP16*, *NT5C2* and *CCDC68* genes have been identified in a recent mega-analysis of genome-wide association studies. To date, the role of these SNPs on gray matter (GM) volumes remains unclear.

Methods: After performing quality control for minor-allele frequency > 5% using a JPT HapMap sample and our sample, a genotyping call rate > 95% and Hardy-Weinberg equilibrium testing ($p > 0.01$), five of eight SNPs were eligible for analysis. We used a comprehensive voxel-based morphometry (VBM) technique to investigate the effects of these five SNPs on GM volumes between major-allele homozygotes and minor-allele carriers in Japanese patients with schizophrenia ($n = 173$) and healthy subjects ($n = 449$).

Results: The rs7914558 risk variant at *CNNM2* was associated with voxel-based GM volumes in the bilateral inferior frontal gyri (right $T = 4.96$, $p = 0.0088$, left $T = 4.66$, $p = 0.031$). These peak voxels, which were affected by the variant, existed in the orbital region of the inferior frontal gyri. Individuals with the risk G/G genotype of rs7914558 had smaller GM volumes in the bilateral inferior frontal gyri than carriers of the non-risk A-allele. Although several effects of the genotype and the genotype-diagnosis interaction of other SNPs on GM volumes were observed in the exploratory VBM analyses, these effects did not remain after the *FWE*-correction for multiple tests ($p > 0.05$).

Conclusions: Our findings suggest that the genetic variant in the *CNNM2* gene could be implicated in the pathogenesis of schizophrenia through the GM volumetric vulnerability of the orbital regions in the inferior frontal gyri.

Keywords: Schizophrenia, Genome-wide association study, Voxel-based morphometry, Cyclin M2 (*CNNM2*), Inferior frontal gyrus

Background

Schizophrenia is a common and complex psychiatric disorder with a lifetime risk of approximately 1%. This disorder has a strong genetic component; indeed, the estimated heritability is 81% [1]. Multiple genetic variants that have a small effect have been implicated in the pathogenesis of schizophrenia [2]. A genome-wide association study (GWAS) of single-nucleotide polymorphisms (SNPs) that accesses tens of thousands of DNA samples from patients and controls can be a powerful tool for identifying common risk factors for complex diseases, such as schizophrenia. To date, GWASs on schizophrenia have identified several genome-wide significant associated variants located in the zinc finger protein 804A (*ZNF804A*), neurogranin (*NRGN*), transcription factor 4 (*TCF4*) genes and a major histocompatibility complex (MHC) region [3,4]. Subsequently, the influences of these SNPs in the genes on brain function and structure have been reported [5]. We have found that the genome-wide supported variant of the *NRGN* gene is associated with the brain morphology of the anterior cingulate cortex in patients with schizophrenia [6].

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Recently, a combining analysis of a mega-analysis of GWAS data from 17 separate studies (9,394 cases and 12,462 controls) and the replication data (8,442 cases and 21,397 controls) of European ancestry have found genome-wide significant associations of schizophrenia with eight SNPs [rs7914558 (cyclin M2; *CNNM2*), rs1625579 (micro-RNA 137, *MIR137*), rs17662626 (PCGEM1, prostate-specific transcript, *PCGEM1*), rs2021722 (tripartite motif containing 26, *TRIM26*), rs10503253 (CUB and Sushi multiple domains 1, *CSMD1*), rs7004633 (matrix metallopeptidase 16, *MMP16*), rs11191580 (5′-nucleotidase, cytosolic II, *NT5C2*) and rs12966547 (coiled-coil domain containing 68, *CCDC68*)] from the five new (1p21.3, 2q32.3, 8p23.2, 8q21.3 and 10q24.32-q24.33) and two previously reported (6p21.32-p22.1 and 18q21.2) loci [7]. In studies exploring brain activation during a sentence completion task, individuals without the risk allele of the *MIR137* genotype had significantly greater activation in the posterior right medial frontal gyrus than at-risk individuals [8]. To our knowledge, however, no study has investigated the effects of these SNPs on gray matter (GM) volumes.

Many attempts have been made to minimize the clinical and genetic heterogeneity in studies of schizophrenia. One strategy for gene discovery uses neurobiological quantitative traits (QT) as intermediate phenotypes that reflect the underlying genetic vulnerability better than diagnostic categorization, such as schizophrenia [9,10]. This strategy has the potential to reduce clinical and genetic heterogeneity [11]. Structural GM volumes indicate substantial heritability rates ranging from moderate (40–70%) to high (70–95%) in the frontal and temporal brain regions [12,13]. Mata analyses of brain morphological studies in individuals with first-episode schizophrenia and neuroleptic naive schizophrenia as well as chronic patients with schizophrenia revealed reduction of GM volume in frontal, striato-limbic and temporal regions were present in the early stage of schizophrenia and were unrelated to the effects of neuroleptic treatment, chronicity and duration of illness [14-16]. Some studies have shown that abnormalities in GM volumes are intermediate phenotypes that bridge the gap between the genotype and diagnostic categorization [11,17]. Characterizing the functional effects of novel and poorly understood genetic variants on the intermediate phenotypes provides important insights into the neural mechanisms by which the variants increase the risk for schizophrenia [18]. Our research group has had a long-standing interest in the effects of genetic variants (i.e., *COMT, DISC1, PACAP, BDNF, APOE, AKT1* and *NRGN*) on brain structure in psychiatric disorders [6,19-24]. In this study, we examined the impact of the genome-wide supported variants on the GM volumes of patients with schizophrenia and healthy subjects. Using a comprehensive voxel-based morphometry (VBM) technique, we tested the hypothesis that these risk variants would be associated with GM volumes.

Material and methods

Subjects

VBM analyses were conducted on 173 patients with schizophrenia (59.0% males, 102 males and 71 females, mean age ± SD, range 36.0 ± 12.3 years) and 449 healthy subjects (47.7% males, 214 males and 235 females, mean age ± SD, range 35.4 ± 12.8 years). All subjects were biologically unrelated within the second-degree of relationship and were of Japanese descent [25,26]. 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. Patients were recruited from the Osaka University Hospital. Each patient with schizophrenia had been diagnosed by at least two trained psychiatrists according to the criteria from 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, the healthy subjects 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 and handedness did not differ significantly between the study group and the controls ($p > 0.38$), while the female ratio, years of education, estimated premorbid intelligence quotient (IQ) and total gray matter volumes were significantly lower in the patients with schizophrenia compared to the controls ($p < 0.016$) (Additional file 1: Table S1). All participants provided written informed consent after the study procedures had been fully explained. This study was performed in accordance with the World Medical Association's Declaration of Helsinki and was approved by the Research Ethical Committee of Osaka University.

SNP selection and SNP genotyping

We selected eight SNPs, rs7914558, rs1625579, rs17662626, rs2021722, rs10503253, rs7004633, rs11191580 and rs12966547, from a previous mega analysis of GWASs [7]. Of these eight SNPs, rs17662626 in the *PCGEM1* gene (2q32.3) was not polymorphic in the samples obtained from the HapMap Japanese in Tokyo (JPT) project. Because the other seven SNPs were common genetic variants in the HapMap JPT samples (minor allele frequency > 5%), we focused on these SNPs. Venous blood was collected

from the subjects, and genomic DNA was extracted from whole blood according to standard procedures. These SNPs were genotyped using the TaqMan 5′-exonuclease allelic discrimination assay (Assay ID: rs7914558: C__31978821_10, rs1625579: C___8946584_20, rs2021722: C__11690541_10, rs10503253: C___1503810_20, rs7004633: C__29048976_10, rs11191580: C__31656012_10 and rs12966547: C____152930_10, Applied Biosystems, Foster City, CA, USA), as previously described [19,22]. Detailed information on the PCR conditions is available upon request. The genotyping call rates were 98.2% (rs7914558), 97.9% (rs1625579), 83.6% (rs2021722), 97.9% (rs10503253), 97.9% (rs7004633), 99.8% (rs11191580) and 97.6% (rs12966547). No deviation from the Hardy-Weinberg equilibrium (HWE) in the examined SNPs was detected in the patients or in controls ($p > 0.01$), with the exception of rs2021722. A significant deviation from HWE in the rs2021722 was found in both the patients ($p = 1.02 \times 10^{-17}$) and controls ($p = 2.05 \times 10^{-36}$) with a relative excess of CC homozygotes, TT homozygotes and undetermined subjects. According to the dbSNP database (National Center for Biotechnology Information), the SNP is shown as a tri-allelic variant with T/C/A. A number of genome-wide significant variants within MHC (6p21.32-p22.1), including rs2021722, have been identified [7]. However, the MHC region has been excluded from further analysis. Analyzing the region is difficult because of its high linkage disequilibrium (LD) and ethnic heterogeneity. Minor allele frequencies of rs1625579 were under 5% in our patients (3.2%) and controls (2.5%). Therefore, in this study, we excluded these SNPs rs2021722 and rs1625579 from the VBM analyses. Genotype and allele distributions for each SNP included in the VBM analyses between the patients with schizophrenia and the controls are shown in Table 1. All risk alleles were defined based on the previous GWAS [7]: rs7914558 (major G-allele), rs10503253 (minor A-allele), rs7004633 (minor G-allele), rs11191580 (major T-allele) and rs12966547 (minor G-allele). To increase the statistical power and decrease type I errors, homozygotes and the heterozygotes for the minor allele groups were combined and treated as minor-allele carriers. In this study, we contrasted GM volumes between minor allele carriers and major allele homozygotes.

Magnetic resonance imaging procedure

All magnetic resonance imaging (MRI) studies were performed on a 1.5 T GE Signa 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). Subjects with MRI abnormalities, such as infarcts, hemorrhages or brain tumors, were screened out prior to including this study as part of routine clinical diagnosis and treatment. Therefore, there were no gross abnormalities in any of the subjects. Each image was visually examined to eliminate images with motion or metal artifacts, and the anterior commissure-posterior commissure line was adjusted. The MRI images were processed using the VBM8 toolbox in Statistical Parametric Mapping 8 (SPM8; http://www.fil.ion.ucl.ac.uk/spm/software/spm8/) running on MATLAB R2013a (MathWorks, Natick, MA, USA) according to the VBM8-Toolbox Manual (http://dbm.neuro.uni-jena.de/vbm8/VBM8-Manual.pdf). The T1 images were normalized and segmented into GM, white matter (WM) and cerebrospinal fluid (CSF) using the VBM8 toolbox with defaults for the extended options. The modulated non-linear only (i.e., with no affine component) option was selected to create volumetric GM partitions. Finally, the images were smoothed with an 8-mm full-width, half-maximum isotropic Gaussian kernel.

Statistical analyses

In genetic association analysis, we performed power calculations using the Power Calculator for Two-Stage Association Studies (http://www.sph.umich.edu/csg/abecasis/CaTS/) [28]. The power estimate was based on an allele frequency of 0.53 at rs7914558, a prevalence of 0.01, an alpha level of 0.05, and assuming varying degrees of odds ratio using a multiplicative model. In brain morphological analyses, we performed power

Table 1 Genotype and allele distributions for each SNP between the patients with schizophrenia and healthy subjects

			Risk allele	Genotype frequencies						Risk allele frequencies		Allelic p value	OR (95% CI)
				+/+	+/−	−/−	+/+	+/−	−/−				
SNP IDs	Gene	Chr	+/−	SCZ ($n = 173$)			CON ($n = 449$)			SCZ	CON	($\chi 2$)	
rs10503253	*CSMD1*	8p23.2	A/C	0.06	0.46	0.49	0.09	0.46	0.45	0.29	0.32	0.22 (1.5)	1.19 (0.90-1.56)
rs7004633	*MMP16*	8q21.3	G/A	0.03	0.37	0.60	0.06	0.36	0.57	0.21	0.25	0.24 (1.4)	1.20 (0.89-1.61)
rs7914558	*CNNM2*	10q24.32	G/A	0.23	0.53	0.24	0.29	0.49	0.22	0.49	0.53	0.23 (1.4)	1.16 (0.91-1.49)
rs11191580	*NT5C2*	10q24.33	T/C	0.49	0.42	0.09	0.50	0.42	0.07	0.70	0.72	0.55 (0.4)	1.09 (0.83-1.43)
rs12966547	*CCDC68*	18q21	G/A	0.15	0.44	0.41	0.17	0.44	0.39	0.37	0.39	0.54 (0.4)	1.09 (0.84-1.41)

Abbreviations: *Chr* Chromosome; *SCZ* patients with schizophrenia; *CON* healthy controls; +, risk allele; -, non-risk allele; *OR* odds ratio.
For alleles, the first allele is the risk allele. All risk alleles are represented based on the previous GWAS [7].

calculations using the G*Power Version 3.1.5 [29]. The power estimate was based on an alpha level of 0.05, a power of 0.80 and assuming varying degrees of effect size using *t* tests. Standardized effect sizes were calculated using Cohen's *d* method (http:www.uccs.edu/faculty/lbecker).

Statistical analyses of the demographic variables were performed using PASW Statistics 18.0 software (SPSS Japan Inc., Tokyo, Japan). Based on the assumption that most of demographic variables, such as age and education years, were not fitted to a normality distribution with the Kolmogorov-Smirnov test ($p < 0.05$), differences in clinical characteristics between patients and controls or between genotypes were analyzed using the non-parametric Mann–Whitney *U*-test for continuous variables, such as age and years of education, and χ^2 tests for categorical variables, such as gender and handedness, as shown in Additional file 1: Table S1-S3. The presence of HWE was examined using the χ^2 test for goodness-of-fit via SNPAlyze V5.1.1 Pro software (DYNACOM, Yokohama, Japan). The allelic distributions of each SNP between patients and controls were analyzed using χ^2 tests with the SNPAlyze software. The significance levels for HWE and other statistical tests were set at two-tailed *p*-values, $p < 0.01$ and $p < 0.05$, respectively.

We performed a comprehensive exploratory whole brain search using the SPM8 statistical tools to examine the effects of the diagnosis, the genotype and their interaction of each SNP on GM volume in patients with schizophrenia and healthy subjects. As two-way ANOVA can simultaneously investigate these effects only in one model, these effects on GM volume were statistically assessed using a full factorial model for a 2 × 2 ANOVA with diagnosis (cases and controls) and genotype status (major-allele homozygotes and minor-allele carriers) as independent variables in SPM8. Gray matter volumes are correlated with age, and gender and years of education differed significantly between the patient and the control groups (Additional file 1: Table S1). Therefore, age, gender and years of education were included as covariates of no interest into the analyses to control for confounding variables. We contrasted GM volumes between the diagnostic groups (smaller volume region in patients with schizophrenia compared with healthy subjects), the genotype groups (smaller or larger volume region in minor-allele carriers relative to major-allele homozygotes) or the genotype-diagnosis interaction. Non-sphericity estimation was used. We applied a voxel-level height threshold of $p < 0.001$ (uncorrected for multiple comparisons) and clusters of more than 100 contiguous voxels were considered for the exploratory VBM analyses. And then we applied family-wise error (*FWE*) correction for multiple testing to avoid type I errors at the whole brain level. Eventually, the significance level was set at $p < 0.05$ ($FWE\text{-}_{corrected}$). To obtain a cluster as large as possible, we extracted relative GM volumes from nominal clusters in bilateral inferiror frontal gyri at the lenient uncorrected threshold of $p < 0.001$ and cluster sizes > 100. The extraction of these relative GM volumes were performed after including confounding factors such as age, sex and education years and modulated by total brain volumes in the VBM analyses. Anatomic localization was performed according to both the MNI coordinates and Talairach coordinates, which were obtained from M. Brett's transformations (http://imaging.mrc-cbu.cam.ac.uk/imaging/MniTalairach) and presented as Talairach coordinates.

Results

Our study size of 173 cases and 449 controls had sufficient power (>80%) to detect a genetic effect at odds ratio of 1.43 or greater for rs7914558 when the allele frequency was 0.53. Unfortunately, in our sample sizes, there was no allelic association with schizophrenia for any of the five SNPs [rs7914558 (*CNNM2*), rs10503253 (*CSMD1*), rs7004633 (*MMP16*), rs11191580 (*NT5C2*) and rs12966547 (*CCDC68*)] ($p > 0.22$, Table 1).

We investigated the effects of diagnosis (cases and controls), genotype (major-allele homozygotes and minor-allele carriers) and their interaction of five SNPs on GM volumes in a comprehensive exploratory VBM analysis. The effects of diagnosis between patients with schizophrenia and healthy subjects were found in all analyses of the present study ($p < 0.05$). Patients with schizophrenia showed smaller GM volumes compared with healthy subjects primarily in the frontal and temporal lobes, including the bilateral inferior frontal gyri, which was consistent with previous studies [14,30]. We found significant effects for the risk-allele homozygotes of rs7914558 on decreased GM volume in the bilateral inferior frontal gyri (right, $T = 4.96$, $p = 0.0088$; left, $T = 4.66$, $p = 0.031$), as shown in Table 2 and the regions based on the hot color map in Figure 1. To compare the effects of the genotype in both the patients with schizophrenia and healthy subjects, we extracted the means and SD for relative GM volumes from nominal clusters in bilateral inferior frontal gyri and the extracted GM volumes were shown in Figure 2 and Additional file 1: Table S2. The risk G-allele homozygotes of the *CNNM2* polymorphism had smaller GM volumes in the bilateral inferior frontal gyri compared to the non-risk allele carriers. The inferior frontal gyrus can be subdivided into three macroanatomical structures: the orbital (Brodmann area: BA47), opercular (BA44) and triangular (BA45) parts. In the present study, the peak GM regions affected by the *CNNM2* genotype existed in the orbital parts of the bilateral inferior frontal gyri. When the two genotype groups (G-allele homozygotes and A-allele carriers) were divided into three genotype groups (individuals with G/G genotype, G/A genotype and A/A genotype) and we performed an additional VBM analysis using a multiple regression model, the number of risk G-allele was significantly

Table 2 Effects of the *CNNM2* genotype and genotype-diagnosis interaction on GM volumes

Brain regions	R/L	BA	CS	*p* values (peak)		Talairach coordinates		
				T	*FWE*	*x*	*y*	*z*
Non-risk minor allele carrier > Risk major allele homozygote								
Inferior frontal Gyrus	R	11/47	1306	4.96	**0.0088**	22	31	−20
Inferior frontal Gyrus	L	47	437	4.66	**0.031**	−22	18	−22
Middle frontal Gyrus	L	11	667	4.33	0.11	−24	38	−18
Posterior cingulate	L	29	248	3.73	0.61	−7	−48	11
Non-risk minor allele carrier < Risk major allele homozygote								
no suprathreshold clusters								
Genotype-diagnosis interaction								
Superior temporal Gyrus	L	22	598	4.23	0.16	−46	−20	1

Abbreviations: *R* right; *L* left; *BA* Brodmann area; *CS* Cluster size; *FWE* family-wise error.
All regions shown have nominal association at a voxel-level height threshold of $_{uncorrected}$ $p < 0.001$ and a minimum clusters extent of 100 voxels. Significant results ($FWE\text{-}_{corrected}$ $p < 0.05$) are shown in bold face.

related to smaller GM volumes of the right inferior frontal gyrus ($T = 4.67$, $p = 0.029$) but not the left inferior frontal gyrus ($T = 3.64$, $p = 0.70$) in total subjects.

As shown in Additional file 1: Table S1, patients with schizophrenia participated in this study were chronic and the symptoms were moderately stable. There was no difference in demographic information, such as duration of illness or PANSS scores, between risk G-allele homozygotes and non-risk A-allele carriers of rs7914558 ($p > 0.20$, Additional file 1: Table S3). However, to find whether there was potential clinical impact on our outcomes, we additionally investigated the genotype effect on GM volume including chlorpromazine equivalent of total antipsychotics (mg/day), duration of illness or PANSS scores as covariant for VBM analyses only in patients. The genotype effect on these regions did not change even after controlling for these factors, suggesting that there was no potential clinical impact on our outcomes.

In the exploratory VBM analyses, we also found nominal effects of the risk-allele homozygotes of rs7914558 on decreased GM volume in the left middle frontal gyrus and the left posterior cingulate and a nominal genotype-diagnosis interaction with the GM volume in the left superior temporal gyrus ($_{uncorrected}$ $p < 0.001$, Table 2).

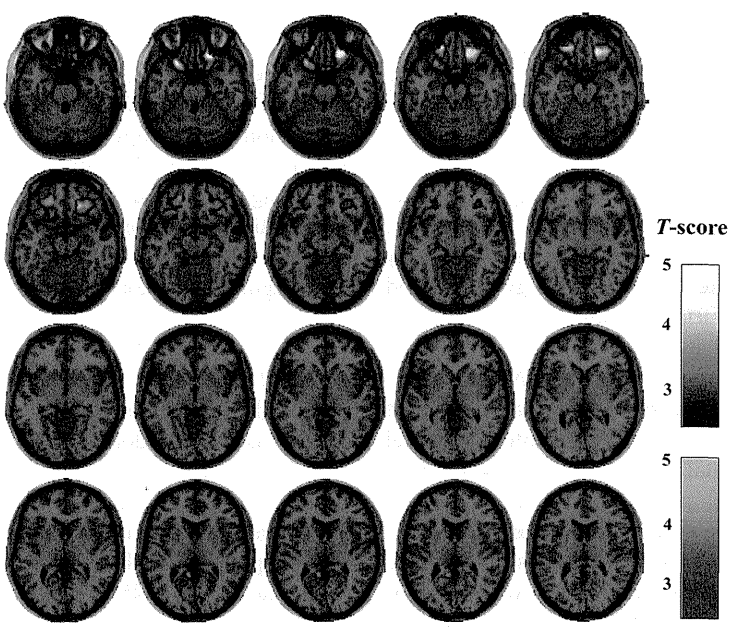

Figure 1 Effects of the rs7914558 polymorphism at the *CNNM2* gene on GM volumes. There were effects of the risk-allele carriers of rs7914558 at *CNNM2* on decreased GM regions (red areas shown on the hot color map). There was no effect of the genotype on increased GM regions (blue area shown on the winter color map). Each color map shows the *t* values corresponding to the color in the figure.

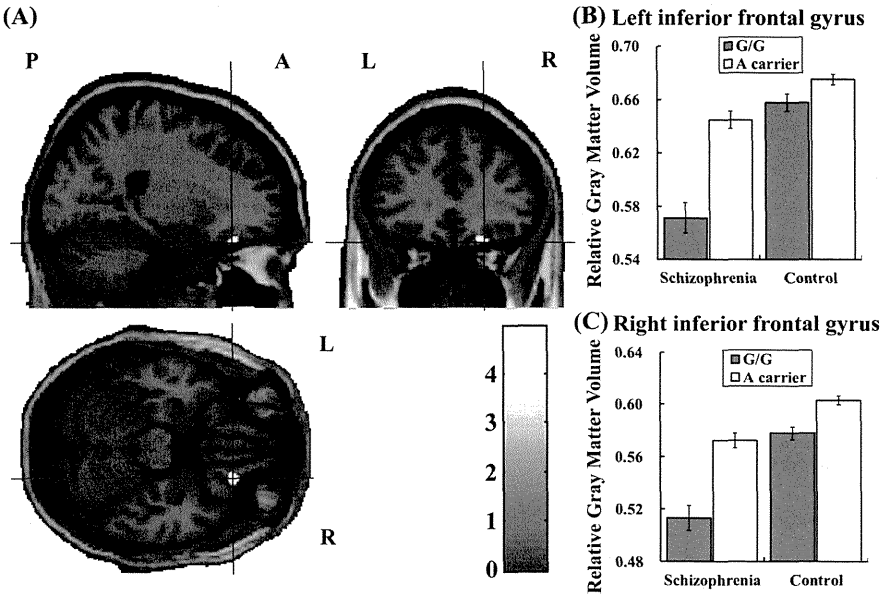

Figure 2 The impacts of the *CNNM2* genotype on GM volume of the bilateral inferior frontal gyri. (A) Anatomical localizations are displayed on coronal, sagittal, and axial sections of a normal MRI spatially normalized into the Montreal Neurological Institute template (p <0.05). The most significant cluster of the genotype effect was in the right inferior frontal gyrus. The region is shown as a cross-hairline. The color bars show the t values corresponding to the color in the figure. **(B, C)** Each column shows the relative GM volumes extracted from a nominal cluster at $_{uncorrected}$ $p < 0.001$ and cluster size > 100 in the right inferior frontal gyrus (peak Talairach coordinates; 22, 31, -20) **(B)** and in the left inferior frontal gyrus (peak Talairach coordinates; -22, 18, -22) **(C)**. Error bars represent the standard error.

Additionally, we found several marginal effects of genotypes and genotype-diagnosis interactions of other SNPs on GM volumes in the exploratory analyses ($_{uncorrected}$ $p < 0.001$, Additional file 1: Table S4 and Figure S1-S4). However, these effects of genotypes and genotype-diagnosis interactions on GM regions did not survive after the *FWE*-correction for multiple tests at the whole brain level ($p > 0.05$).

Discussion

To date, it remains unclear whether the genome-wide significant risk variants for schizophrenia in a mega-analysis of GWASs influenced GM volumes. This study is the first to identify the GM morphology associated with genome-wide risk variants using a comprehensive VBM technique. Of the five genetic variants investigated in this study, we found influences of the *CNNM2* genotype on the bilateral inferior frontal gyri at the whole brain level. GM volumes in the bilateral inferior frontal gyri, particularly the orbital region, in the risk G-allele homozygotes of *CNNM2* polymorphism were smaller than those observed in the non-risk allele carriers.

In genetic association analysis, our sample size had sufficient power (>80%) to detect a genetic effect with odds ratio of 1.43 or greater for rs7914558. However, previous large GWAS has reported the genetic effect with low odds ratio of 1.10 for the SNP [7]. To detect such a small genetic effect, our sample size had insufficient power (12%), and a large sample size of at least 3400 patients and 3400 controls is needed. On the other hand, in brain morphological analyses, our sample size had sufficient power (>80%) to detect a genotype effect on GM volumes at medium effect size (Cohen's *d)* of 0.25 or greater. The observed effect sizes on the inferior frontal gyri were medium to large (0.29-1.00). These findings suggest that the genome-wide supported variant of schizophrenia had larger effect on GM volumes of the inferior frontal gyri than diagnostic status, and support that GM volumes abnormalities were prominent intermediate phenotypes bridging the gap between a susceptibility genetic variant and diagnostic categorization.

Rs7914558 is located in intron1 of the *CNNM2* gene (also known as *ACDP2*) on chromosome 10q24.32. The *CNNM2* gene spans 160.3 kb of genomic DNA and contains eight exons. This gene belongs to a member of the ancient conserved domain-containing protein family because the protein shares a domain conserved in a large number of species ranging from bacteria to human [31]. Members of this protein family contain a sequence motif that is present in the cyclin box, a cyclic nucleotide-monophosphate (cNMP)-binding domain. The *CNNM2* gene has a ubiquitous expression pattern in humans [31]. In particular, the level of *CNNM2* expression in the brain is moderate to high (http://www.ebi.ac.uk/gxa/experiment/E-MTAB-37/ENSG00000148842).

However, whether the expression level of this gene in the brains of patients with schizophrenia is lower or higher than that in healthy subjects is unknown. The encoded protein CNNM2 plays an important role in magnesium homeostasis by modulating Mg2+ concentration. The *CNNM2* mRNA is upregulated when there is a deficiency of magnesium in the brain [32]. CNNM2 mediated Mg2 + −sensitive Na + currents were blocked by increased extracellular Mg2+ concentrations [33]. We assessed the effect of the rs7914558 genotype on *CNNM2* expression using bioinformatics data (http://www.sanger.ac.uk/resources/software/genevar/[34]) to examine whether the rs7914558 genotype might be an expression quantitative trait loci (eQTL). *In silico* analysis showed that the *CNNM2* gene expression of the high-risk G genotype of rs7914558 was significantly lower than that of the non-risk genotype in the combined lymphoblast-derived HapMap CEU and YRI samples ($r = -.23$, $t = -2.59$, $p = 0.011$). The low expression of this gene resulted increased Mg2+ levels. Increased extracellular Mg2+ concentrations caused a decrease in the activity of the glutamate *N*-methyl-D-aspartate (NMDA) receptor [35]. These findings suggest that the *CNNM2* gene may play an important role in the hypofunction of the NMDA receptor, which is implicated in the pathophysiology of schizophrenia.

The inferior frontal gyrus has a multifunctional role in human behavior, interpersonal interactions and communication [36]. The inferior frontal gyrus consists primarily of the heteromodal association neocortex, which is a major site of involvement in schizophrenia [37]. Several studies have reported that the relative GM in patients with schizophrenia was significantly reduced in the bilateral inferior frontal areas [38-40]. The inferior frontal gyrus can be subdivided into three macroanatomical parts: orbital, opercular and triangular. The orbital region is one of the major regions of the social brain that connects to the orbitofrontal cortex [41], while the opercular and triangular regions form Broca's area, which is an important region for speech-language production [42]. We found that the *CNNM2* genotype affects brain volumes in the orbital regions of the inferior frontal gyri. Functionally, the orbital region is thought to be involved in the processing of empathy [36] and sentence comprehension [43,44] in the left hemisphere and decision-making cognition [41] and fine movement control [36] in the right hemisphere. Social functions are widely impaired in patients with schizophrenia [45-49]. It is still unclear whether and to what extent the effects of *CNNM2* polymorphism on GM structure observed here might be associated with an increased risk for schizophrenia. We suggest that the *CNNM2* variant may play a role in the social cognition and social functioning impairments noted in patients with schizophrenia through GM volumetric vulnerability of the orbital regions of the inferior frontal gyri. Further research is needed to investigate how a possible relationship between the *CNNM2* gene and hypofunction of the NMDA receptor would result in decreased GM volumes of the inferior frontal gyri.

There was no significant effect of the other four variants on any GM volumes. There are several possible reasons for the absence of an association. A false negative association cannot be excluded in our study because we applied a strict *FWE* correction for multiple comparisons at the whole brain level ($p < 0.05$). In the Additional file 1: Figure S1-S4, the regions shown at the more lenient uncorrected threshold of $p < 0.001$ may be helpful in further studies. Interestingly, many of the effects of these genome-wide significant variants at the lenient level involved decreased GM volumes, including the medial, middle and inferior fontal gyri. Reduced GM volumes in these regions have been repeatedly demonstrated in imaging studies of schizophrenia [14,30]. Another interpretation is that the effect of these variants was not sufficiently sensitive to the morphological vulnerability of the GM volumes. The effect of these variants may be preferable in identifying genotype-related vulnerability on other intermediate phenotypes, such as cognitive functions and personality traits. Therefore, further research is needed to confirm whether the effects of these variants could be related to the susceptibility of cognitive functioning.

There were several limitations to this study. Because a number of statistical analyses, including the effects of diagnosis, genotypes and their interaction on GM volumes, were performed, a correction for multiple testing should be considered. However, a consensus on how to correct such multiple testing on study combining brain imaging and genetics has not been reached in this research field. To control type I errors, we applied the strict *FWE* correction for all VBM analyses, while we did not perform any correction on the genetic modality. The existence of a false positive association cannot be excluded as an explanation for our results, although we were quite careful to match ethnicity and correct for multiple testing. Further investigations of other samples with much larger sample sizes and/or with different ethnicities and/or in relatives of those with schizophrenia are needed to confirm our findings. It is unclear whether our results are directly/indirectly linked to the *CNNM2* polymorphism rs7914558, to other polymorphisms in high LD with this variant or to interactions between this variant and other variants. To determine whether rs7914558 is the most strongly associated variant for schizophrenia and brain structure in this gene, an extensive search for other functional variants at this locus is needed. Additionally, as with other risk variants for schizophrenia, clarifying the biological role of this SNP through *in vitro* and *in vivo* studies is important to improve the understanding of the pathophysiology of schizophrenia.

Conclusions

We found that a genome-wide supported variant of *CNNM2* could be associated with GM morphological vulnerability of the bilateral inferior frontal gyri. These results suggest that there may be possible deleterious effects of the risk G-allele at *CNNM2* in the inferior frontal gyri, which may, at least partially, represent the mechanism by which *CNNM2* increases the risk for schizophrenia. Further research will be needed to clarify the function of the risk *CNNM2* variant on the pathophysiology of schizophrenia.

Additional file

Additional file 1: Table S1. Demographic information for patients with schizophrenia and healthy subjects. **Table S2:** Effects of the rs7914558 genotype on extracted relative GM volumes of the bilateral inferior frontal gyri. **Table S3:** Demographic information for risk G-allele homozygotes and non-risk A-allele carriers of rs7914558. **Table S4:** Impacts of each genetic variant on GM volumes in the exploratory VBM analyses. **Figure S1:** Effects of the rs10503253 polymorphism (*CSMD1*) on GM volumes. **Figure S2:** Effects of the rs7004633 polymorphism (*MMP16*) on GM volumes. **Figure S3:** Effects of the rs11191580 polymorphism (*NT5C2*) on GM volumes. **Figure S4:** Effects of the rs12966547 polymorphism (*CCDC68*) on GM volumes.

Abbreviations

GM: Gray matter; VBM: Voxel-based morphometry; GWAS: Genome-wide association study; SNP: Single-nucleotide polymorphism; *CNNM2*: Cyclin M2; QT: Quantitative traits; DSM-IV: Diagnostic and statistical manual of mental disorders fourth edition; SCID: Structured clinical interview for DSM-IV; HWE: Hardy-Weinberg equilibrium; LD: Linkage disequilibrium; MRI: Magnetic resonance imaging; FWE: Family-wise error.

Competing interests

The authors declare that they have no competing interests.

Authors' contributions

RH supervised the entire project, collected the data, wrote the manuscript, was critically involved in the design, analysis and interpretation of the data and was responsible for performing the literature review. KO was critically involved in the collection and analysis of the data, and contributed to the editing of the final manuscript and contributed intellectually to the interpretation of the data. HY and SU took part in genotyping. HY, YY, MF, MF, YW, MI, HK and MT contribute with sample collection and gave comments to the manuscript. All authors contributed to and have approved the final manuscript.

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ORIGINAL ARTICLE

Influence of the *NRGN* gene on intellectual ability in schizophrenia

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Genome-wide association studies have reported an association between schizophrenia and rs12807809 of the *neurogranin* (*NRGN*) gene. We have recently found that an rs12807809–rs12278912 haplotype of the gene is associated with schizophrenia in a Japanese population and that the *NRGN* expression of the high-risk TG haplotype is lower than that of the protective TA haplotype in immortalized lymphoblasts. In this study, we investigated the influences of *neurogranin* genotypes (rs12807809 and rs12278912), haplotypes and diplotypes and genetic variant–diagnosis interactions on intellectual ability in 414 Japanese patients with schizophrenia and healthy subjects. We detected possible effects of the genome-wide screen-supported rs12807809, haplotypes, diplotypes and their genetic variant–diagnosis interactions on intellectual abilities at the threshold level of $P<0.05$. After applying Bonferroni correction for 13 genotype measures and setting *P*-values for significance ($P<0.0039$; 0.05/13), three effects remained significant: the rs12807809–rs12278912 diplotype–diagnosis interactions on performance intelligence quotient (CG/CG: $P=3.9\times10^{-13}$; TA/TA: $P=1.1\times10^{-7}$) and TA/TA diplotype on performance intelligence quotient in patients with schizophrenia ($P=8.2\times10^{-8}$) remained significant. The intellectual abilities of the high-risk TG/TG diplotype of the *neurogranin* gene were lower compared to those with the non-risk TA/TA diplotype. Our findings suggest that the genetic risk variant in the *neurogranin* gene may be related to reduced intellectual ability.
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Keywords: diplotype; GWAS; *NRGN*; performance IQ; schizophrenia

INTRODUCTION

Schizophrenia is a common and complex psychiatric disease with strong genetic components. Schizophrenia has an estimated heritability of approximately 80%,[1,2] and many genes have been implicated in the pathogenesis of schizophrenia.[3]

Three genome-wide association studies (GWAS) and follow-up case–control studies have reported seven single-nucleotide polymorphisms (SNPs) in combined samples of 12 945 patients with schizophrenia and 34 591 controls of European Caucasian ethnic background.[4] Of the seven SNPs, only one SNP, rs12807809 of the *neurogranin* (*NRGN*) gene, was common in the HapMap Japanese samples in Tokyo as previous reported.[5] The frequency of the T allele of rs12807809 was higher in individuals with schizophrenia than in those without the disorder in both the original study (odds ratio = 1.15)[4] and recent follow-up study (odds ratio = 1.12).[6] We have recently found using a gene-based approach that the rs12807809–rs12278912 haplotype was the variant near the *NRGN* gene most strongly associated with schizophrenia in a Japanese population.[5] Moreover, we have found that the *NRGN* expression of the high-risk TG haplotype of rs12807809–rs12278912 was significantly lower than the expression of the protective TA haplotype in immortalized lymphoblasts derived from the HapMap Japanese samples in Tokyo samples and our Japanese case–control samples.[5] The *NRGN* gene on chromosome 11q24.2 spans 7.3 kb of genomic DNA and contains four exons.[7] *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.[8] NRGN has an important role in the Ca^{2+}–CaM signaling pathway.[9] Ca^{2+} influx-induced oxidation of NRGN leads to postsynaptic activation of CaM-dependent protein kinase II by CaM, which is associated with strengthened *N*-methyl-D-aspartate receptor signaling.[10] Reduced function of *NRGN* is considered to mediate the effects of the *N*-methyl-D-aspartate hypofunction implicated in the pathophysiology of schizophrenia.

NRGN is abundantly expressed in the brain regions involved in cognitive functioning and especially enriched in CA1 pyramidal

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neurons in the hippocampus.[11] *NRGN* has been shown to have a role in dendritic spine formation, synaptic plasticity, long-term potentiation and spatial learning.[12,13] *Nrgn* knockout mice displayed deficits in spatial learning and anxiety-like tendencies, supporting a role for *nrgn* in the hippocampus-mediated interaction between stress and performance.[14] The SNP rs12807809 was associated with diminished hippocampal activation during a contextual fear conditioning task.[15] On the other hand, reduced NRGN immunoreactivity has been observed in Brodmann areas 9 and 32 of the prefrontal cortex in post-mortem brains from patients with schizophrenia.[16] We have found that carriers of the risk allele of rs12807809 (T) had a smaller gray matter volume in the left anterior cingulate cortex (Brodmann area 32) than carriers of the non-risk allele (C) in patients with schizophrenia.[17] It has been reported that rs12807809 is associated with differential neural activation in the anterior and posterior cingulate cortices during episodic memory encoding and retrieval tasks.[18] These results suggest that the *NRGN* gene is related to the function of the hippocampus and anterior cingulate and that dysfunction of the gene leads to neurocognitive deficits in patients with schizophrenia.

Intelligence quotient (IQ) is a standardized measure of human intellectual capacity that takes into account a wide range of cognitive skills.[19] The intellectual ability of patients with schizophrenia is lower than healthy subjects. Approximately 50% of patients with schizophrenia show cognitive deterioration, with an IQ decline of 10 points from the premorbid IQ.[20] The declined IQ in schizophrenia remains stable, although there is considerable interindividual variation in the degree of decline.[21] Intellectual dysfunction in unaffected relatives of schizophrenia patients is similar to but somewhat less pronounced than that in patients with schizophrenia.[22] The estimated heritability of IQ is high in the general population (69–85%) and individuals with familial schizophrenia (64–74%).[23,24] Schizophrenia and IQ are related and both highly heritable, but their genetic overlap is controversial.[25–27] It has been reported that there is a low phenotypic correlation between premorbid IQ and psychosis[25] vs the high correlation between postmorbid IQ and schizophrenia.[27] To a greater or lesser extent, some susceptibility genes for schizophrenia would mediate liability for the disorder at least partly by influencing intellectual abilities. Although two studies have investigated the association of the genome-wide screen-supported rs12807809 with IQ in three Caucasian populations,[28,29] these studies reported no association between rs12807809 and IQ in the populations. So far, however, no study has investigated the effect of the rs12807809 on IQ in a Japanese population. Moreover, although we have previously reported that the rs12807809–rs12278912 haplotype is associated with risk for schizophrenia and the *NRGN* expression,[5] no study has investigated the effects of *NRGN* genetic variants, including haplotypes and diplotypes, except for the rs12807809 on intellectual abilities. In this study, we used IQ to find downstream effects of these genetic variants (rs12807809 and rs12278912), the rs12807809–rs12278912 haplotype and rs12807809–rs12278912 diplotype of the *NRGN* gene in Japanese patients with schizophrenia and healthy volunteers.

MATERIALS AND METHODS

Subjects

This study was conducted with 157 patients with schizophrenia (52.2% males (82 males and 75 females); mean age ± s.d., 37.1 ± 12.4 years) and 257 healthy subjects (41.6% males (107 males and 150 females); mean age ± s.d., 37.5 ± 12.0 years). All subjects were biologically unrelated within the second-degree of relationship and of Japanese descent.[30,31] All subjects (100%) and the majority of subjects (79.2%) in this study have been included the previous *NRGN* genetic association and imaging genetic studies, respectively.[5,17] Subjects were excluded if they had neurological or medical conditions that could potentially affect the central nervous system, such as atypical headache, previous 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 Osaka 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 received psychiatric medication. The mean age and sex ratio did not differ significantly between cases and controls ($P>0.036$), whereas the years of education and estimated premorbid IQ were significantly lower in the patients with schizophrenia than in the controls ($P<0.0039$) (Supplementary Table S1). When the three genotypes of either rs12807809 or rs12278912 were compared, we found no differences in demographic variables ($P>0.020$) (Supplementary Table S1). Written informed consent was obtained from all subjects after the procedures had been fully explained. This study was conducted in accordance with the World Medical Association's Declaration of Helsinki and approved by the Research Ethical Committee of Osaka University.

SNP selection and genotyping

We selected rs12807809 and rs12278912 of the *NRGN* gene for genotyping as described in the introduction. The rs12807809 is located on 3457 bases upstream of the *NRGN* gene and the rs12278912 is located in intron 1 of the gene. The T/C polymorphism rs12807809 and G/A polymorphism rs12278912 have been described previously in the GWAS and our studies.[4,5,17] An rs12807809–rs12278912 haplotype is a combination of the two alleles at adjacent loci on 11q24.2 that are inherited together. An rs12807809–rs12278912 diplotype is a combination of the haplotypes for each individual. 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 (Assay ID: rs12807809; C_32029000_20, rs12278912; C_32029002_10; Applied Biosystems, Foster City, CA, USA) as described previously.[32,33] Detailed information on the polymerase chain reaction conditions is available upon request. No deviation from the Hardy–Weinberg equilibrium was detected in the examined SNPs in the patients or controls ($P>0.05$).

Measurement of intellectual abilities and assessment of current symptoms of schizophrenia

To assess intellectual abilities, we used the full-scale IQ, which is divided into performance IQ and verbal IQ, of the Japanese version of the Wechsler Adult Intelligence Scale-revised or third edition.[34] The subjects were assessed by trained clinical psychologists to obtain full-scale, performance and verbal IQ scores on the Wechsler Adult Intelligence Scale. Current symptoms of schizophrenia were evaluated using the positive and negative syndrome scale.[35]

Statistical analyses

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 using PASW Statistics 18.0 software (SPSS Japan, Tokyo, Japan). Deviation from the Hardy–Weinberg equilibrium was tested separately in test cases and controls using χ^2 tests for goodness of fit using SNPAlyze V.5.1.1 Pro software (Dynacom, Yokohama, Japan).

The effects of diagnosis, *NRGN* genotype and their interaction on intellectual abilities were analyzed by two-way analyses of covariance. Diagnosis and genotype status were included in the analysis as independent variables. Full-scale, performance and verbal IQ scores were included as

dependent variables. As intellectual abilities may be influenced by sex and years of education, these variables were corrected for as covariates. We did not include age as a covariate because IQ scores were already corrected for age.

HPlus (http://qge.fhcrc.org/hplus) is a software application for estimating haplotype/diplotype frequencies, inferring individual haplotypes/diplotypes based on expectation-maximization and progressive ligation algorithms,[36] and assessing haplotypic/diplotypic associations with various phenotypes using linear regression. The minimum frequency for a haplotype or diplotype to be estimated for association was 1% of patients and controls. The differences in intellectual abilities between patients with schizophrenia and healthy subjects or among haplotypes or diplotypes were analyzed using logistic regression or linear regression with HPlus. We also examined interactions of haplotype–diagnosis or diplotype–diagnosis using linear regression. Each genotype was treated as the number of major alleles (0, 1 or 2) in the analysis. For joint haplotype analysis in HPlus, each haplotype or diplotype was tested against the reference haplotype or diplotype (the most frequent haplotype or diplotype). Sex (1: male; 2: female), years of education and diagnosis (0: controls; 1: patients) were corrected for in these analyses as covariates. We applied Bonferroni correction in the all statistical tests, based on the number of 13 genotype measures; SNPs (two), haplotypes (four haplotypes minus a reference haplotype) and diplotypes (nine diplotypes minus a reference diplotype). The significance level for all statistical tests was finally set at two-tailed $P<0.0039$ (0.05/13).

RESULTS

Impact of genetic variants of the *NRGN* gene on intellectual abilities

First, we investigated the effects of diagnosis, *NRGN* genotype (genome-wide supported rs12807809 and rs12278912) and their interaction on full-scale IQ (Table 1). We found significant effects of diagnosis ($P<2.95\times10^{-9}$) and a possible effect of genotype of rs12807809 ($P=0.017$) on full-scale IQ. As expected, patients with schizophrenia showed significantly lower IQ than healthy subjects in all analyses of this study. There was no effect of diagnosis–genotype interactions or rs12278912 genotype on full-scale IQ ($P>0.14$). Second, we investigated the effects of diagnosis and genotype and their interaction on performance and verbal IQ, respectively. We found significant effects of diagnosis ($P<4.12\times10^{-6}$) and possible effects of rs12807809 genotype ($P=0.017$) and diagnosis–rs12807809 interactions ($P=0.040$) on performance IQ. Full-scale and performance IQ have a tendency to be lower in individuals with the major risk T allele of rs12807809 than those with the minor C allele. There was no effect of rs12278912 or diagnosis–rs12278912 interactions on performance IQ or genotype or diagnosis–genotype interaction on verbal IQ ($P>0.058$).

Impact of rs12807809–rs12278912 *NRGN* haplotype on intellectual abilities

Based on the effect of genotype, we next investigated the effects of diagnosis, rs12807809–rs12278912 haplotype and their interaction on full-scale IQ and performance IQ (Table 2). These intellectual scores were lower in patients with schizophrenia than healthy subjects ($z>-11.2$, $P<3.3\times10^{-14}$). We found a subtle effect of diagnosis–CA haplotype interactions on performance IQ ($z=2.3$, $P=0.022$). When we further explored the effect of haplotype on performance IQ separately in patients with schizophrenia and healthy subjects, there was a subtle effect of the CA haplotype on performance IQ in patients with schizophrenia ($z=2.2$, $P=0.026$). There was no effect of the CA haplotype in healthy subjects ($z=-1.2$, $P=0.21$). Although there was a subtle association, the performance IQ of the high-risk TG haplotype (reference haplotype) of rs12807809–rs12278912 has a tendency to be lower than that of the CA haplotype in patients with schizophrenia. There was no effect of other haplotypes or diagnosis–haplotype interactions on performance IQ or any haplotype or diagnosis–haplotype interactions on full-scale IQ ($P>0.11$).

Impact of rs12807809–rs12278912 *NRGN* diplotype on performance IQ

Based on the effect of haplotype, we further investigated the effects of diagnosis, rs12807809–rs12278912 diplotype and their interaction on performance IQ (Table 3). As described above, the intellectual scores were lower in patients with schizophrenia than healthy subjects. We found two significant diagnosis–diplotype interactions (CG/CG: $P=3.9\times10^{-13}$; TA/TA: $P=1.1\times10^{-7}$) and four possible effects of diagnosis–diplotype interactions (CA/TG: $P=0.022$; TA/CG: $P=0.022$) and diplotypes (CA/TA: $P=0.048$; TA/TA: $P=0.039$) on performance IQ. Other diplotypes and interactions had no effect on performance IQ ($P>0.11$). Because we found two significant diagnosis–diplotype interactions on performance IQ, we separately examined the effect of diplotype on performance IQ in patients with schizophrenia and healthy subjects. There was a diplotype with significant effect on performance IQ in patients with schizophrenia (TA/TA: $P=8.2\times10^{-8}$), whereas there were five diplotypes with possible effects on performance IQ in patients with schizophrenia (CA/TG: $P=0.035$; TA/CG: $P=0.035$; CA/CG: $P=0.013$) and in healthy controls (CA/TA: $P=0.044$; TA/TA: $P=0.036$). The performance IQ of the TG/TG diplotype group was significantly lower than that of the TA/TA diplotype group of patients with schizophrenia.

Table 1 Impact of genetic variants in the *NRGN* gene on intellectual function

	Schizophrenia (N = *157*)			*Control* (N = *257*)			P-*values* (F_{2406}-*values*)		
Variables	*M/M*	*M/m*	*m/m*	*M/M*	*M/m*	*m/m*	*Diagnosis*	*Genotype*	*Interaction*
rs12807809[a]	T/T (*N*=91)	T/C (*N*=62)	C/C (*N*=4)	T/T (*N*=142)	T/C (*N*=94)	C/C (*N*=21)			
Full-scale IQ	84.2±17.7	88.2±18.2	101.5±10.7	108.5±11.9	109.7±13.0	111.0±8.2	**$\underline{2.95\times10^{-9}}$ (36.8)**	0.017 (4.1)	0.14 (2.0)
Performance IQ	80.1±16.3	85.2±18.1	99.3±10.8	107.6±11.4	107.3±13.0	108.7±11.4	**$\underline{8.11\times10^{-11}}$ (44.6)**	0.017 (4.1)	0.040 (3.3)
Verbal IQ	90.3±17.5	92.3±17.7	102.8±8.2	107.7±13.1	109.9±13.3	112.8±11.7	**$\underline{4.12\times10^{-6}}$ (21.8)**	0.052 (3.0)	0.28 (1.3)
rs12278912	G/G (*N*=107)	G/A (*N*=45)	A/A (*N*=5)	G/G (*N*=157)	G/A (*N*=87)	A/A (*N*=13)			
Full-scale IQ	85.2±18.3	88.6±17.1	87.4±19.5	109.7±11.6	109.0±12.7	102.5±11.9	**$\underline{6.16\times10^{-12}}$ (50.2)**	0.37 (1.0)	0.23 (1.5)
Performance IQ	81.4±17.4	84.9±16.7	88.0±20.2	108.4±11.4	107.4±12.9	99.1±10.2	**$\underline{2.32\times10^{-12}}$ (52.4)**	0.55 (0.6)	0.058 (2.9)
Verbal IQ	90.7±18.0	93.2±16.1	88.6±21.8	109.1±12.6	109.2±14.0	104.5±13.3	**$\underline{2.25\times10^{-8}}$ (32.5)**	0.31 (1.2)	0.67 (0.4)

Abbreviations: IQ, intellectual quotient; M, major allele; m, minor allele; NRGN, neurogranin; SNP, single-nucleotide polymorphism.
Means±s.d. are shown. To control for confounding factors, the effects of diagnosis, *NRGN* genotype and their interaction on IQ were analyzed by two-way analyses of covariance, with sex and years of education as covariates. Significant *P*-values ($P<0.0039$) are shown in boldface and underlined.
[a]The genome-wide supported SNP for schizophrenia.[4]

Table 2 Association between rs12807809 and rs12278912 haplotype and performance IQ

					P-*values (z-scores)*	
Haplotypes	*Frequency (%)*	*Coefficient*	*s.e.*	*CI*	*Haplotype effect*	*DH interaction*
Total subjects (N = *414*)						
TG	61.5	0 (ref.)	—	—	—	
CG	18.3	0.93	1.49	(−1.99 to 3.84)	0.53 (0.6)	0.59 (0.5)
TA	13.7	−2.16	1.36	(−4.82 to 0.50)	0.11 (−1.6)	0.46 (0.7)
CA	6.6	−2.66	2.08	(−6.73 to 1.41)	0.20 (−1.3)	0.022 (2.3)
Schizophrenia (N = *157*)						
TG	66.2	0 (ref.)	—	—	—	
CG	16.3	3.51	3.37	(−3.09 to 10.11)	0.30 (1.0)	
TA	11.5	1.20	2.96	(−4.60 to 7.01)	0.69 (0.4)	
CA	6.0	9.81	4.40	(1.19 to 18.43)	0.026 (2.2)	
Healthy control (N = *257*)						
TG	58.5	0 (ref.)	—	—	—	
CG	19.5	0.92	1.59	(−2.19 to 4.02)	0.56 (0.6)	
TA	15.0	−2.14	1.49	(−5.05 to 0.78)	0.15 (−1.4)	
CA	7.0	−2.90	2.33	(−7.47 to 1.67)	0.21 (−1.2)	

Abbreviations: CI, confidence interval; DH interaction, diagnosis–haplotype interaction; IQ, intelligence quotient; s.e., standard error.
Joint Association Analysis (the reference haplotype is the most frequent TG haplotype). For the joint haplotype test, several haplotypes were tested against the reference TG haplotype using linear regression analysis. There was a significant effect of diagnosis ($z = -11.2$, $P = 1.1 \times 10^{-14}$). There was no significant *P*-value ($P < 0.0039$).

DISCUSSION

In this study, we investigated the impacts of *NRGN* SNPs, haplotypes and diplotypes and genetic variant–diagnosis interactions on intellectual abilities that are known to be impaired in schizophrenia, in 157 patients with schizophrenia and 257 healthy subjects. After correction for multiple tests, we have provided evidence for the rs12807809–rs12278912 diplotype–diagnosis interactions. There was a significant effect of the diplotype of the *NRGN* gene on performance IQ in patients with schizophrenia but not in healthy subjects. The risk variant of the *NRGN* gene was associated with low intellectual ability.

To examine the association between the genome-wide screen-supported rs12807809 of the *NRGN* gene and intellectual ability (verbal, performance and full-scale IQ), this study was conducted on a Japanese population of patients with schizophrenia and healthy subjects. Thus far, two studies have investigated the association of the SNP with intellectual ability and reported no association between the rs12807809 and any IQ. Donohoe *et al.*[28] investigated the association between the SNP and general cognitive ability (verbal, performance and full-scale IQ) in 393 Irish patients with schizophrenia or schizoaffective disorder and 157 controls, and follow-up samples of 240 German patients and 1344 healthy participants. Krug *et al.*[29] investigated the association between the SNP and verbal IQ using 521 healthy subjects with Western and Middle European descent. We also did not find a significant association between the SNP and intellectual abilities in Japanese subjects, consistent with the previous studies in the different Caucasian populations.[28,29] These findings suggest that the *NRGN* polymorphism may not have a major role in the intellectual abilities.

This report is the first investigation of the association of haplotypes and diplotypes of the *NRGN* gene with intellectual abilities in patients with schizophrenia and healthy subjects. We have determined that the frequencies of the major TG and TA haplotypes of rs12807809–rs12278912 were higher and lower, respectively, in patients with schizophrenia compared with healthy controls.[5] In addition, we have found that *NRGN* gene expression of the high-risk TG haplotype was significantly lower than that of the protective TA haplotype in lymphoblasts. According to these findings, we hypothesized that the IQ of the high-risk TG haplotype group would be lower than that of the protective TA group. However, this prediction was not confirmed in this study, suggesting that the haplotype of the *NRGN* gene may not have a major role in contributing to the intellectual impairments. Instead, we determined that the performance IQ of the major TG/TG diplotype was lower than that of the TA/TA diplotype. When we examined the exploratory association between *NRGN* diplotype and schizophrenia using a Japanese sample (2019 schizophrenia patients and 2574 controls) included in previous study,[5] the ratio of the frequency of the TA/TA diplotype vs the major TG/TG diplotype of rs12807809–rs12278912 in patients (0.020/0.388) was lower compared with controls (0.028/0.349, odds ratio = 0.65, $P = 0.036$). These findings suggest that the performance IQ of subjects with the high-risk TG/TG diplotype was lower compared with the protective TA/TA diplotype in schizophrenia. Expression assay of haplotypes in our previous study[5] was performed using lymphoblasts but not brain tissues. Although the sample sizes of the associated diplotypes are small and limited, further research investigating an association between the diplotype and RNA expression data derived from lymphoblastic cell lines and several tissues including brain is required to enhance our findings.

There are several limitations to interpreting our results. Whether this study has adequate statistical power to detect genetic effects is important. Power calculation was performed using the G*Power 3 program.[37] In the power calculation, our sample size had power >80% to detect an effect size index f of >0.152 among genotype groups with $\alpha = 0.05$. Effect sizes are typically categorized as small ($f = 0.10$), medium ($f = 0.25$) or large ($f = 0.40$). A false-negative association could not be excluded in our study because the effect size index f for genotype on intellectual function was 0.143. Because a number of statistical analyses (64 tests), including the effects of diagnosis, genetic variants of genotype (two SNPs), haplotypes (four haplotypes) and diplotypes (nine diplotypes) and their interaction on

Table 3 Association between rs12807809–rs12278912 diplotype and performance IQ

					P-values (z-scores)	
Diplotypes	*Frequency (%)*	*Coefficient*	*s.e.*	*CI*	*Diplotype effect*	*DD Interaction*
Total subjects (N = 414)						
TG/TG	37.9	0 (ref.)	—	—	—	—
CG/TG	22.7	2.52	1.99	(−1.38 to 6.41)	0.21 (1.3)	0.80 (−0.3)
TA/TG	16.4	0.08	2.05	(−3.94 to 4.10)	0.97 (<0.1)	0.88 (−0.2)
CA/TG	7.9	−1.45	2.32	(−5.99 to 3.09)	0.53 (−0.6)	0.022 (2.3)
TA/CG	4.9	−1.45	2.32	(−5.99 to 3.09)	0.53 (−0.6)	0.022 (2.3)
CG/CG	3.1	−1.14	4.26	(−9.50 to 7.21)	0.79 (−0.3)	**3.9×10^{-13} (7.3)**
CA/CG	2.7	5.01	3.11	(−1.09 to 11.11)	0.11 (1.6)	0.11 (1.6)
CA/TA	2.2	−10.93	5.52	(−21.74 to 0.12)	0.048 (−2.0)	0.32 (1.0)
TA/TA	1.9	−5.39	2.61	(−10.50 to 0.28)	0.039 (−2.1)	**1.1×10^{-7} (5.3)**
CA/CA	0.2	—	—	—	—	—
Schizophrenia (N = 157)						
TG/TG	42.0	0 (ref.)	—	—	—	
CG/TG	25.5	0.73	3.33	(−5.80 to 7.25)	0.83 (0.2)	
TA/TG	14.6	−1.06	3.53	(−7.98 to 5.86)	0.76 (−0.3)	
CA/TG	8.2	8.61	4.08	(0.61 to 16.61)	0.035 (2.1)	
TA/CG	3.9	8.61	4.08	(0.61 to 16.61)	0.035 (2.1)	
CG/CG	0.6	—	—	—	—	
CA/CG	1.9	14.01	5.61	(3.02 to 25.00)	0.013 (2.5)	
CA/TA	1.9	1.70	12.49	(−22.78 to 26.18)	0.89 (0.1)	
TA/TA	1.3	11.64	2.17	(7.39 to 15.90)	**8.2×10^{-8} (5.4)**	
CA/CA	0	—	—	—	—	
Healthy control (N = 257)						
TG/TG	35.4	0 (ref.)	—	—	—	
CG/TG	21.0	2.33	1.99	(−1.57 to 6.23)	0.24 (1.2)	
TA/TG	17.5	−0.05	2.06	(−4.09 to 4.00)	0.98 (<0.1)	
CA/TG	7.7	−1.57	2.35	(−6.18 to 3.04)	0.50 (−0.7)	
TA/CG	5.5	−1.57	2.35	(−6.18 to 3.04)	0.50 (−0.7)	
CG/CG	4.7	−1.17	4.11	(−9.22 to 6.89)	0.78 (−0.3)	
CA/CG	3.1	4.85	3.14	(−1.30 to 11.01)	0.12 (1.5)	
CA/TA	2.3	−10.97	5.44	(−21.63 to 0.31)	0.044 (−0.2)	
TA/TA	2.3	−5.51	2.63	(−10.66 to 0.36)	0.036 (−2.1)	
CA/CA	0.4	—	—	—	—	

Abbreviations: CI, confidence interval; DD interaction, diagnosis–diplotype interaction; IQ, intelligence quotient; s.e., standard error.
The minimum frequency for a diplotype to be estimated for association was 1% of patients or controls. Joint Association Analysis (the reference diplotype is the most frequent TG/TG diplotype). For the joint diplotype test, several diplotypes were tested against the reference TG diplotype using linear regression analysis. There was a significant effect of diagnosis ($z = -11.2$, $P = 1.1 \times 10^{-14}$). Significant *P*-values ($P < 0.0039$) are shown in boldface and underlined.

intellectual abilities (three IQs), were performed, a correction for multiple testing should be considered. However, a consensus on how to correct such multiple testing has not been reached in this research field. Because all tests including genetic variants (genotypes, haplotypes and diplotypes) and each IQ (full-scale, performance and verbal) were not independent and several hypotheses were included, we applied the Bonferroni correction for all statistical tests based on the numbers of 13 genotype measures (genotypes, haplotypes and diplotypes). Because of applying the methods of correcting such multiple testing, we cannot exclude the possibility of false-positive results. As there was no evidence for a specific dominant or recessive model (homozygous allele carriers vs homozygous carriers of other one or two alleles; comparison of two genotype groups) of genetic variants in *NRGN*, our analysis was based on the comparison of three genotype groups. The reason why the diplotype but not the SNPs or the haplotype of the *NRGN* gene was associated with IQ is unclear. Because our results were based on a relatively small number of individuals with the TA/TA diplotype of rs12807809–rs12278912, a future replication study using larger sample sizes is needed to confirm our findings.

In this study, we found an effect of *NRGN* genetic variant on intellectual ability. Our results support an association between the *NRGN* gene and schizophrenia and a hypothesis that the *NRGN* gene may mediate the risk associated with schizophrenia via intellectual dysfunction.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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Plasma levels of mature brain-derived neurotrophic factor (BDNF) and matrix metalloproteinase-9 (MMP-9) in treatment-resistant schizophrenia treated with clozapine☆

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HIGHLIGHTS

- Plasma levels of mature BDNF in schizophrenia were measured for the first time.
- No significant difference was observed in mature BDNF levels in schizophrenia.
- MMP-9 plasma levels were significantly increased in patients with schizophrenia.
- Plasma mature BDNF levels were significantly correlated with plasma MMP-9 levels.

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ABSTRACT

Brain-derived neurotrophic factor (BDNF) regulates the survival and growth of neurons, and influences synaptic efficiency and plasticity. Peripheral BDNF levels in patients with schizophrenia have been widely reported in the literature. However, it is still controversial whether peripheral levels of BDNF are altered in patients with schizophrenia. The peripheral BDNF levels previously reported in patients with schizophrenia were total BDNF (proBDNF and mature BDNF) as it was unable to specifically measure mature BDNF due to limited BDNF antibody specificity. In this study, we examined whether peripheral levels of mature BDNF were altered in patients with treatment-resistant schizophrenia. Matrix metalloproteinase-9 (MMP-9) levels were also measured, as MMP-9 plays a role in the conversion of proBDNF to mature BDNF. Twenty-two patients with treatment-resistant schizophrenia treated with clozapine and 22 age- and sex-matched healthy controls were enrolled. The plasma levels of mature BDNF and MMP-9 were measured using ELISA kits. No significant difference was observed for mature BDNF however, MMP-9 was significantly increased in patients with schizophrenia. The significant correlation was observed between mature BDNF and MMP-9 plasma levels. Neither mature BDNF nor MMP-9 plasma levels were associated clinical variables. Our results do not support the view that peripheral BDNF levels are associated with schizophrenia. MMP-9 may play a role in the pathophysiology of schizophrenia and serve as a biomarker for schizophrenia.

Abbreviations: BDNF, brain-derived neurotrophic factor; MMP-9, matrix metalloproteinase-9; MDD, major depressive disorder; DSM-IV, diagnostic and statistical manual of mental disorders, fourth edition; PANSS, positive and negative syndrome scale.

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1. Introduction

Schizophrenia is a severe psychiatric disease characterized by delusions, hallucinations, impairment of cognitive function and incoherent behavior. It affects approximately 1% of the general population worldwide. Mounting evidence suggests that a deficit in neurotrophin supply to cortical neurons may be an underlying factor in the pathophysiology of schizophrenia as adequate neurotrophic support is required for normal brain development, maturation and function [3,4].

Brain-derived neurotrophic factor (BDNF) is a neurotrophin that regulates neuronal survival, differentiation and growth during brain development, with important effects on neurogenesis and neuroplasticity. It is also important for hippocampal-related learning and memory [17]. A common single nucleotide polymorphism (SNP) of the BDNF gene has impact on episodic memory, hippocampal morphology and memory-related hippocampal activity in human [9,16]. Mature BDNF is initially synthesized as a precursor protein, preproBDNF. Following cleavage of the signal peptide, proBDNF is converted to mature BDNF by extracellular proteases, such as matrix metalloproteinase-9 (MMP-9). Mature BDNF and poBDNF each plays important roles in several physiological functions. Recent studies show that mature BDNF and pro BDNF elicit opposing effects via the TrkB and $p75^{NTR}$ receptors respectively. Mature BDNF preferentially binds to the TrkB receptor and plays an important role through BDNF-TrkB signaling which fulfills wide variety of functions such as cell survival, migration, outgrowth of neuritis and synaptic plasticity. In contrast, pro BDNF preferentially binds to the $p75^{NTR}$ receptors and elicit apoptosis rather than cell survival [8,11]. Considering the important roles of mature BDNF, it would be informative to specifically measure mature BDNF. Although BDNF levels in human blood can be measured using commercially available human BDNF ELISA kits, due to the limited specificity of the BDNF antibody, it has not been possible to distinguish between proBDNF and mature BDNF. Recently, peripheral levels of mature BDNF have been reported to be measurable using newly available human BDNF ELISA kits [23].

It is of great interest to assess the potential contribution of BDNF to the pathophysiology of schizophrenia. Several studies report altered BDNF mRNA and protein in prefrontal cortical regions and hippocampus of post-mortem brain tissues [13,21,22]. Peripheral BDNF levels in patients with schizophrenia have also been widely reported in the literature. However, there is no widespread agreement on the degree of peripheral BDNF levels in patients with schizophrenia, as measured in blood serum or plasma. A recent meta-analysis reported that peripheral BDNF levels were reduced in schizophrenia. However, there was considerable heterogeneity in the results [5]. Considering the important roles of mature BDNF such as cell survival, migration, outgrowth of neuritis and synaptic plasticity, it would be informative to specifically measure mature BDNF in patients with schizophrenia because dysfunction of these mature BDNF roles might be an underlying factor in the pathophysiology of schizophrenia. The peripheral BDNF levels previously reported in patients with schizophrenia were total BDNF (proBDNF and mature BDNF); peripheral levels of mature BDNF specifically have not been investigated in patients with schizophrenia. This study aimed to determine whether peripheral levels of mature BDNF were altered in patients with treatment-resistant schizophrenia. We also investigated Matrix metalloproteinase-9 (MMP-9) levels, as MMP-9 plays a role in the conversion of proBDNF to mature BDNF [8].

Table 1
Demographic variables for subjects.

Variables	Control $n=22$	Patients with schizophrenia $n=22$
Age (years)	38.1 ± 12.9	38.1 ± 13.2
Gender (male/female)	(12/10)	(12/10)
Age at onset	–	21.9 ± 8.4
Duration of illness	–	17.2 ± 11.1
PANSS positive	–	23.0 ± 4.6
PANSS negative	–	25.5 ± 5.5
PANSS general	–	52.9 ± 9.6
Clozapine dose (mg)	–	448.6 ± 130.0

Means ± SD are shown.

2. Materials and methods

2.1. Subjects

Twenty-two patients with treatment-resistant schizophrenia who were treated with clozapine were included in this study. Twenty-two age- and sex-matched healthy controls also participated in this study (Table 1). Cases were recruited at Osaka University hospitals. Each 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 structured clinical interview. Treatment-resistant schizophrenia was defined according to the following criteria mentioned in clozapine drug information in Japan: (1) Non- or little response to treatment from at least two adequately dosed antipsychotic trials for at least 4 weeks (including at least one second-generation antipsychotic, >600 mg/day of chlorpromazine equivalent) and patients never had the Global Assessment of Functioning (GAF) scores that were higher than 40. (2) Intolerance to at least two second-generation antipsychotics because of uncontrolled extrapyramidal symptoms. All subjects included in this study met the criteria of non- or little response. Symptoms of schizophrenia were assessed using the Positive and Negative Syndrome Scale (PANSS). 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 and Chiba University.

2.2. Measurement of mature BDNF and MMP-9

Plasma levels of mature BDNF and MMP-9 were measured using the human BDNF ELISA Kit (Adipo Bioscience, Santa Clara, CA, USA), and the human MMP-9 ELISA Kit (R&D Systems, Minneapolis, MN, USA), respectively. To minimize assay variance, plasma levels of mature BDNF and MMP-9 were measured in each subject on the same day. All experiments were performed in duplicate. Protocols were performed according to the manufacturer's instructions. The optical density of each well was measured using an automated microplate reader (Emax; Molecular Devices, Sunnyvale, CA, USA). As plasma levels of proBDNF are not measurable by the newly available proBDNF ELISA kit due to low sensitivity, we measured only mature BDNF.

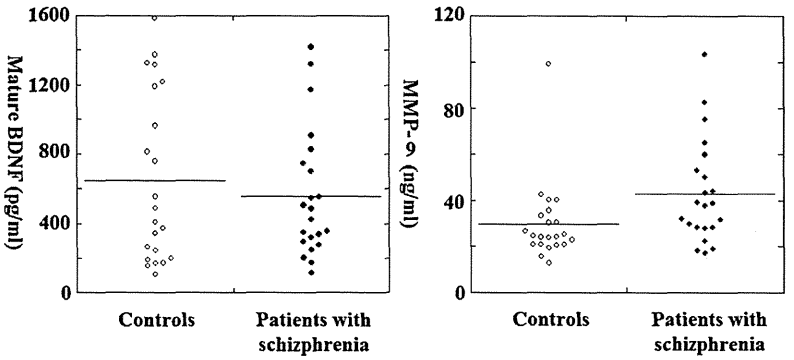

Fig. 1. Plasma levels of mature BDNF and MMP-9 in treatment-resistant schizophrenia treated with clozapine. The plasma levels of mature BDNF and MMP-9 in the controls and treatment-resistant patients with schizophrenia who were treated with clozapine (control, $n=22$, schizophrenia, $n=22$).

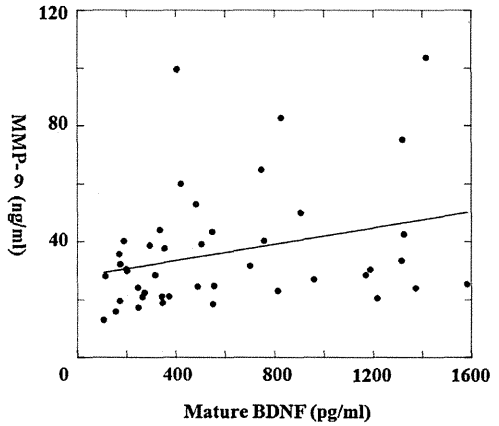

Fig. 2. Correlation between plasma levels of mature BDNF and MMP-9. Positive correlation was observed between plasma levels of mature BDNF and MMP-9 (patients with schizophrenia and controls, $n=44$, $r=0.333$, $p=0.027$).

2.3. Statistical analysis

Statistical analyses were performed using SPSS 20.0J software (SPSS Japan Inc., Tokyo, Japan). Differences in clinical characteristics between patients and controls were analyzed using χ^2 tests for categorical variables. The groups did not differ with respect to age or gender (Table 1). Kolmogrov–Smirnov test was used to test the normality of data distribution. Mature BDNF did not normally distribute in both patients with schizophrenia and controls (patients with schizophrenia; $p=0.041$, controls; $p=0.042$). MMP-9 distributed normally in patients with schizophrenia, however did not distribute normally in controls (patients with schizophrenia; $p=0.130$, controls; $p=0.012$). And differences between patients and controls were analyzed using the Mann–Whitney U-test for continuous variables. Homogeneity of variance was assessed by Levene's test. The assumption of homogeneity of group variance was not violated in both mature BDNF and MMP-9 levels (mature BDNF; $p=0.052$, MMP-9; $p=0.112$). Test of rejection of Smirnoff–Grubbs was performed. Spearman rank order correlation test was performed to assess the possible correlation between plasma levels of mature BDNF and MMP-9 and clinical characteristics. The significant level for statistical tests was set at $p<0.05$.

3. Results

The plasma levels of mature BDNF and MMP-9 were compared between patients with treatment-resistant schizophrenia who were treated with clozapine and controls, and no significant difference was observed for mature BDNF (Fig. 1, Mann–Whitney test; $U=238$, $p=0.925$). However, MMP-9 was significantly increased in patients with schizophrenia (Fig. 1, Mann–Whitney test; $U=139$, $p=0.016$). When we exclude each one sample in both groups by test of rejection of Smirnoff–Grubbs, MMP-9 was still significantly increased in patients with schizophrenia (Mann–Whitney test; $U=118$, $p=0.010$). As MMP-9 plays a role in the conversion of proBDNF to mature BDNF, the correlation between the levels of mature BDNF and MMP-9 was examined. There were significant correlation between the levels of mature BDNF and MMP-9 in (Fig. 2, patients with schizophrenia and controls, $n=44$, $r=0.333$, $p=0.027$). When we investigate this correlation in patients and controls groups separately, significant correlation was observed in patients with schizophrenia ($n=22$, $r=0.585$, $p=0.004$) but not in controls ($n=22$, $r=0.322$, $p=0.143$). To determine the effect of clozapine on mature BDNF and MMP-9 levels, we also examined the correlation between the plasma levels of mature BDNF or MMP-9 and clozapine dosage. No significant correlation was observed between the plasma levels of mature BDNF or MMP-9 and clozapine dosage (Table 2, BDNF and clozapine dosage; $n=22$, $r=0.028$, $p=0.901$, MMP-9 and clozapine dosage; $n=22$, $r=0.131$, $p=0.562$). The correlations between the plasma levels of mature BDNF or MMP-9 and positive and negative symptom scores on the PANSS were also investigated; no significant correlations were observed (Table 2, BDNF and PANSS positive; $n=22$, $r=-0.014$, $p=0.952$, BDNF and PANSS negative; $n=22$, $r=-0.079$, $p=0.726$, MMP-9 and PANSS positive; $n=22$, $r=0.306$, $p=0.167$, BDNF and PANSS negative; $n=22$, $r=0.127$, $p=0.574$). The correlations between the plasma levels of mature BDNF or MMP-9 and duration of illness were also investigated; no significant correlations were observed (Table 2, BDNF and duration of illness; $n=22$, $r=0.121$, $p=0.592$, MMP-9 and duration of illness; $n=22$, $r=0.087$, $p=0.699$).

Table 2
Correlation analysis.

	Clozapine dosage	PANSS positive	PANSS negative	PANSS general	Age at onset	Duration of illness
Mature BDNF	0.901	0.952	0.726	0.865	0.332	0.592
MMP-9	0.562	0.167	0.574	0.454	0.685	0.699

p values are shown.

4. Discussion

In this study, for the first time, we measured the plasma levels of mature BDNF in patients with schizophrenia. The plasma levels of mature BDNF were decreased in treatment-resistant schizophrenia, however the difference did not reach statistical significance. Our result was consistent with some previous studies that investigated the serum levels of total BDNF in patients with schizophrenia [10,20]. Treatment-resistant schizophrenia patients treated with clozapine were enrolled because some studies suggest that peripheral BDNF levels increase in association with antipsychotics treatment including clozapine which is used for the treatment of poorly responsive patients with schizophrenia [6,10] and serum BDNF levels were reported to be significantly correlated with clozapine daily dose but not with typical antipsychotics [15]. However, we found no effect of clozapine treatment on the plasma levels of mature BDNF. A possible explanation would be the difference in race. This is the first study investigating the effect of clozapine treatment on the plasma levels of mature BDNF in Japanese population. Accumulating evidence suggests that BDNF plays a key role in the pathophysiology of major depressive disorder (MDD). It was reported that BDNF serum levels in patients with MDD were significantly lower than those of healthy controls, and that there was a negative correlation between BDNF serum levels and the severity of depression in patients [19]. Furthermore, decreased serum levels of BDNF in antidepressant naive patients with MDD, recovered to levels associated with amelioration of depressive symptoms, after antidepressant treatment. Three meta-analyses and a study using a large sample size confirmed these findings [7]. Recently, peripheral levels of mature BDNF have been reported to be decreased in MDD [23]. Further study using larger samples is needed to see whether peripheral levels of mature BNDF are not altered in schizophrenia and mature BDNF levels are not associated with clozapine.

We also investigated MMP-9 plasma levels, as MMP-9 plays a role in the conversion of proBDNF to mature BDNF [8]. The significant correlation was observed between mature BDNF and MMP-9 plasma levels, suggesting that MMP-9 plays a role in the conversion of proBDNF to mature BDNF in the samples of this study. The serum levels of MMP-9 have been reported to be increased in patients with schizophrenia [2]. A higher frequency of positive MMP-9 activity in serum from patients with schizophrenia has also been reported [1]. We confirmed the presence of elevated plasma MMP-9 levels in patients with treatment-resistant schizophrenia. In patients with schizophrenia, MMP9 might be induced to recover the decreased mature BDNF. The finding that significant correlation between mature BDNF and MMP-9 was observed only in patients with schizophrenia but not in controls supports this idea. Plasma levels of MMP-9 have been proposed to be a useful biomarker for assessing pathological event in brain. It was reported that levels of MMP-9 in plasma and brain were significantly correlated after cerebral ischemia in rats [14]. MMP-9 is an enzyme implicated in a number of pathological conditions including neuropsychiatric disorders [18]. A role of MMP-9 in the plasticity of the central nervous system has been investigated in experimental studies and MMP-9 is reported to be required for hippocampal long-term potentiation and memory [12]. MMP-9 may have some roles in pathophysiology of schizophrenia.

Our study must be interpreted in lights of its limitations. Firstly, the sample size of this study is small. Secondly, only treatment-resistant schizophrenia patients treated with clozapine were included and patients treated with other antipsychotics or patients without antipsychotics treatment were not included in this study. Further studies are needed to evaluate the relationship between plasma levels of mature BDNF and schizophrenia and clozapine treatment.

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