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Variants of the *RELA* Gene are Associated with Schizophrenia and their Startle Responses

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The pathogenesis of schizophrenia is thought to involve aberrant immune and inflammatory responses. Nuclear factor kappa B (NF-κB) has important roles in the immune and inflammatory responses. The *v-rel avian reticuloendotheliosis viral oncogene homolog A* (*RELA*) gene encodes the major component of the NF-κB complex. We genotyped four single-nucleotide polymorphisms (SNPs) in the *RELA* gene and performed a gene-based association analysis using 1224 patients with schizophrenia and 1663 controls. We found significant associations of three SNPs (rs11820062: $p = 0.00011$, rs2306365: $p = 0.0031$, and rs7119750: $p = 0.0080$) with schizophrenia and stronger evidence for association in a multi-marker sliding window haplotype analysis (the lowest $p = 0.00006$). The association between this gene and schizophrenia was evident in male subjects but not in female subjects, when separately analyzed by gender. *In silico* genotype-gene expression analysis using web database and the WGAViewer software revealed that these three schizophrenia-associated SNPs might be related to *RELA* mRNA expression in immortalized B-lymphocytes. *In silico* analysis also suggested the putative promoter SNP, rs11820062, might disrupt the consensus transcription factor binding sequence of the androgen receptor. The impact of four *RELA* polymorphisms on pre-pulse inhibition (PPI) was investigated in 53 patients with schizophrenia. We provided evidence that at risk genotypes of three SNPs were associated with deficits in PPI; however, there was no effect of the one non-risk SNP on PPI. These findings suggest that variants of the *RELA* gene are associated with risk for schizophrenia and PPI deficits in a Japanese population.
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INTRODUCTION

Schizophrenia is a common and complex psychiatric disease. The lifetime morbidity rate is 0.5–1.0% across distinct populations. Family, twin, and adoption studies of schizophrenia have indicated that there are strong genetic factors with an estimated heritability of 80% (Cardno and

Gottesman, 2000; Tsuang, 2000). Although genes implicated in the pathogenesis of schizophrenia have been found through intense research efforts, eg association studies of candidate gene approach, genomewide association studies (GWAS), copy number variation (CNV) studies, and pedigree studies (Harrison and Weinberger, 2005; Cichon et al, 2009), the exact genetic factors of this complex disease remain to be explained. Recent polygenic component analysis in GWAS studies demonstrated the less effect between different populations, eg Europeans and African-Americans, Europeans, and Japanese, compared with the effects between two European populations (Purcell et al, 2009; Ikeda et al, 2010). As it may be due to aggregate differences in allele frequencies and patterns of linkage disequilibrium and/or population specific risk for

schizophrenia, association studies using multiple populations are expected.

Alterations in cytokine expression in schizophrenia have been extensively studied. The levels of cytokines, such as interleukin-1β (IL-1β), IL-1 receptor antagonist (IL-1RA), IL-6, and tumor necrosis factor-α (TNF-α), are increased in the plasma and cerebrospinal fluid of patients with schizophrenia (Naudin *et al*, 1997; Potvin *et al*, 2008). Nuclear factor-kappa B (NF-κB), a prototypical transcription factor, regulates the expression of cytokines. Conversely, NF-κB can be activated by pro-inflammatory cytokines, including IL-1β and TNF-α, and in response to various cell stressors. Many genes have been shown to be responsive to NF-κB (Baeuerle, 1991), including genes involved in survival/apoptosis, immune and inflammatory responses, and cell differentiation. In addition, the NF-κB-responsive genes *IL2*, *IL6*, *TNF-α*, major histocompatibility complex (MHC), Bcl-2 family members, Calbindin, and ICAM-1 have been reported to be associated with schizophrenia (Jarskog *et al*, 2000, 2004; Schwarz *et al*, 2000; Benes *et al*, 2001, 2003; Potvin *et al*, 2008; Woo *et al*, 2008; Purcell *et al*, 2009; Shi *et al*, 2009; Song *et al*, 2009; Stefansson *et al*, 2009).

NF-κB is present in synaptic terminals and serves as a regulator of neuronal plasticity, which is activated by the activity of neuronal circuits (Mattson *et al*, 2000). The NF-κB complex is inhibited by the IκB complex, which inactivates NF-κB by sequestering it in the cytoplasm (Huxford and Ghosh, 2009). After the phosphorylation of serine residues on the IκB proteins, IκB dissociates from and activates the NF-κB complex. The activated NF-κB complex translocates into the nucleus and binds to regulatory elements in target genes. Constitutively, activated NF-κB is detected mostly in glutamatergic neurons, whereas NF-κB in glia has a lower basal activity and is heavily inducible (Kaltschmidt and Kaltschmidt, 2009). Knockout of a subunit of NF-κB or inhibition of NF-κB by super-repressor IκB in neuron of mice resulted in defects in learning and memory and the loss of neuroprotection (Kaltschmidt and Kaltschmidt, 2009). It has been shown that activation of NF-κB prevents neuronal apoptosis in various cell types. H_2O_2 increased NF-κB activation and dopamine D2 receptor expression (Larouche *et al*, 2008), suggesting that NF-κB may participate in the psychopathology of schizophrenia through its effect on the neurotransmitter system. The association between cytokine expression and NF-κB activation has been reported in schizophrenia (Song *et al*, 2009). These findings support the hypothesis that alterations in cytokines and NF-κB, which cause abnormal inflammatory responses, might contribute to the pathogenesis of schizophrenia.

Although three components of NF-κB, NFKB1, NFKB2, and NFKB3, were not in major locus in schizophrenia (OMIM181500: http://www.ncbi.nlm.nih.gov/omim), *NFKB3* located on chromosome 11q13 showed a suggestive linkage to schizophrenia in a family-based linkage disequilibrium analysis in a Japanese population (Yamada *et al*, 2004). The v-rel avian reticuloendotheliosis viral oncogene homolog A (*RELA*) gene (OMIM164014; alternative names include nuclear factor kappa-B, subunit 3 (*NFKB3*), transcription factor NFKB3, NFKB, p65 subunit, and nuclear factor of kappa light chain gene enhancer in

B cells 3) encodes the major subunit of the NF-κB protein complex, are abundantly expressed in neurons and glia (Kaltschmidt and Kaltschmidt, 2009). Mice lacking *RELA* showed a learning deficit in the spatial version of the radial arm maze (Meffert *et al*, 2003), indicating the critical involvement of the *RELA* gene in memory function, which may be related to pathophysiology of memory dysfunction in schizophrenia. Microarray Expression Data in UCSC Genome Bioinformatics Site (http://genome.ucsc.edu/) showed moderate mRNA expression of the *RELA* gene and the components of IκB (*NFKBIA* and *NFKBIB*) in human brain and prominent expression in prefrontal cortex. Altered expression of these genes in postmortem brain of patients with schizophrenia was not found in the pooled gene expression data in the Stanley Genomic Medical Research Institute Online Genomics Database (https://www.stanleygenomics.org). In this study, we first investigated the association between the *RELA* gene and schizophrenia in a Japanese population, and then performed *in silico* genotype–gene expression analysis.

Impaired sensorimotor gating is considered to be a common psychophysiological feature of schizophrenia that could theoretically lead to a variety of severe defects in perception, attention, and thinking (Braff and Geyer, 1990). Pre-pulse inhibition (PPI) of the acoustic startle reflex (ASR) is the most common psychophysiological index of sensorimotor gating. PPI is emerging as an important intermediate phenotype for schizophrenia (Braff and Light, 2005), because it has high heritability (Anokhin *et al*, 2003) and PPI deficits have been found in high-risk subjects (Cadenhead *et al*, 2000). It has been hypothesized that the maternal immune response to infection may influence fetal brain development and lead to schizophrenia (Brown and Derkits, 2010). Prenatal immune challenge by bacterial endotoxin lipopolysaccharide (LPS) or polyriboinosinic–polyribocytidylic acid (poly I : C) resulted in deficits in PPI (Cardon *et al*, 2010; Romero *et al*, 2010). Thus, it has been hypothesized that there are genetic variants that are related to PPI deficits in patients with schizophrenia. Genetic variations in the serotonin-2A receptor, Catechol O-methyltransferase and neuregulin-1 genes have been associated with PPI in schizophrenia (Quednow *et al*, 2008, 2010; Hong *et al*, 2008). Thus, we also analyzed the association between the identified SNPs in the *RELA* gene and PPI in patients with schizophrenia.

MATERIALS AND METHODS

Subjects

The subjects of our genetic association study consisted of 1224 patients with schizophrenia (50. 9% male (623/601), mean age $\pm$ SD: 46.2 $\pm$ 15.0 years) and 1663 healthy controls (46.5% male (773/890), mean age $\pm$ SD: 46.9 $\pm$ 20.7 years). The mean age did not differ significantly between the groups ($Z = -1.10$, $p = 0.27$), while the sex ratio differed significantly between the groups ($\chi^2 = 5.51$, $p = 0.019$). All the subjects were biologically unrelated Japanese individuals. Patients were recruited at the National Center Hospital of Neurology and Psychiatry, Showa University School of Medicine, Fujita Health University School of Medicine and Osaka University Graduate School of

Medicine. Cases were recruited from both outpatients and inpatients at the hospitals. 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), on the basis of unstructured clinical interviews. When the diagnosis of two trained psychiatrists was discordant, they started to discuss the diagnosis. When the disputes about the diagnosis were resolved and the patient was diagnosed as schizophrenia, we included the patients. When the disputes were not resolved by discussion or the patients was not diagnosed as schizophrenia, we excluded the patients. Controls, including hospital and institutional staff, 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, who had received psychiatric medication or who were not Japanese. We did not assess the controls for the family history of mental disorders, such as schizophrenia, bipolar disorder, or major depressive disorder. The ethnicity was determined by the self-report and it was not confirmed by genetic analyses.

Data for the PPI analysis were available for 53 patients with schizophrenia (56.6% males (30/23), mean age ± SD: 39.1 ± 13.2 years). The subjects included in the PPI analysis met additional criteria. All subjects were recruited at Osaka University, and subjects were excluded from this study if they had neurological or medical conditions that could potentially affect the central nervous system, such as atypical headaches, 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. None of the subjects reported history of any known hearing impairment and all participants were able to clearly detect 70 dB noise. Written informed consent was obtained from all subjects after the procedures had been fully explained. This study was performed in accordance with the World Medical Association's Declaration of Helsinki and approved by the institutions' ethical committees (National Center Hospital of Neurology and Psychiatry, Showa University School of Medicine, Fujita Health University School of Medicine and Osaka University Graduate School of Medicine).

SNP Selection and Genotyping

Venous blood was drawn from the subjects, and genomic DNA was extracted from whole blood according to standard procedures. We used the Tagger program (Paul de Bakker, http://www.broad.mit.edu/mpg/tagger) of the Haploview software (ver. 4.1) (Barrett *et al*, 2005) to select SNPs in the HapMap database (release #22/phase II, April 2007, www.hapmap.org, population: Han Chinese in Beijing (CHB) + Japanese in Tokyo (JPT); minor allele frequencies (MAFs) of more than 0.05) that covered the *RELA* gene spanning 8.5 Kb (5′-flanking regions including approximately 2 kb from the first exon and approximately 0.5 kb downstream (3′) from the last exon; HapMap database contig number chr11: 65178000.65189000). The criterion for detecting tag SNPs was an r^2 threshold greater than 0.80 in 'pair-wise tagging only' mode. Two tag SNPs were selected (rs2306365 and rs11820062) by the Tagger program among five SNPs in the HapMap database in the *RELA* gene region. We also searched putative functional SNPs, which are located in exons, exon-intron boundaries and putative promoter regions (5′-flanking region including approximately 2 kb from the first exon and 3′ region approximately 1 kb from first exon). We only find one SNP (rs11820062) fulfilled the criteria, which was already selected by Tagger program. Because these two SNPs were located in the 5′ region, we added two SNPs (rs11568300 and rs7119750) on the 3′ region in this gene for better coverage (2.2 kb per SNP) (Figure 1). The four selected SNPs (rs7119750 (SNP1), rs11568300 (SNP2), rs2306365 (SNP3), and rs11820062 (SNP4)) in the *RELA* gene were genotyped using the TaqMan 5′-exonuclease allelic discrimination assay (Applied Biosystems, Foster City, CA) as described previously (Hashimoto *et al*, 2006, 2007; Ohi *et al*, 2009). The positions of the four SNPs analyzed in the present study are indicated in Figure 1. Primer and probe sequences for detection of the SNPs are as follows: SNP1: forward primer 5′-GCTCAGGCTCAATCCCTCTCTA-3′, reverse primer 5′-CCTACAGGCTGGGTCAGATG-3′, probe 1 VIC-ACTGCCAA CACCC, probe 2 FAM-CACTGCTAACACCC; SNP2: forward primer 5′-GGTGTGCGCAGAGAAGCA-3′, reverse primer 5′-CCTTCTCCATGCAGCTGTCT-3′, probe 1 VIC-CACACT GGCCTCCG, probe 2 FAM-CACAGTGGCCTCCG; SNP3: forward primer 5′-GCCAAGAAAACAGGCGATCAG-3′, reverse primer 5′-CCTCCTCTAGGACTTGTGTTTCAC-3′, probe 1 VIC-CCCTCCCAGTGCAGAG, probe 2 FAM-CCT CCCAGCGCAGAG; SNP4: forward primer 5′-CGCATCTG ATTCAGTTTCCTCTCT-3′, reverse primer 5′-AATCAGG GCCTGTTGTACTTTCTT-3′, probe 1 VIC-CTCCCTCAAT TTTCCT, probe 2 FAM-TCCCTCAGTTTTCCT.

In Silico Analysis to Identify SNPs Associated with *RELA* Expression (eQTLs)

To identify whether the SNPs in the *RELA* gene might be expression quantitative trait loci (eQTLs), we used GeneVar (http://www.sanger.ac.uk/humgen/genevar/). Genotype and gene lymphoblastoid expression data from multiple HapMap ethnic samples such as Japanese, Han Chinese, Utah residents with Northern and Western European ancestry from the CEPH collection, and Yoruban in Ibadan, Nigeria are deposited in GeneVar. Users could not access the original genotype, gene expression and demographic data in each individual; however, users are able to analyze the association between genotype and gene expression by the WGAViewer software as described by the group that developed GeneVar (Stranger *et al*, 2007). WGAViewer software is able to perform correlational analysis with number of allele as a continuous variable (allele dose effect: $1/1 = 0$, $1/2 = 1$, $2/2 = 2$). However, any other statistical models such as categorical analysis such as dominant and recessive are not able to perform by the software. We searched for potential transcription factor binding sites in the sequence that included SNP4 with the Patch 1.0 pattern search program and the TRANSFAC 6.0 public site (http://www.gene-regulation.com/cgi-bin/pub/programs/patch/bin/patch.cgi).
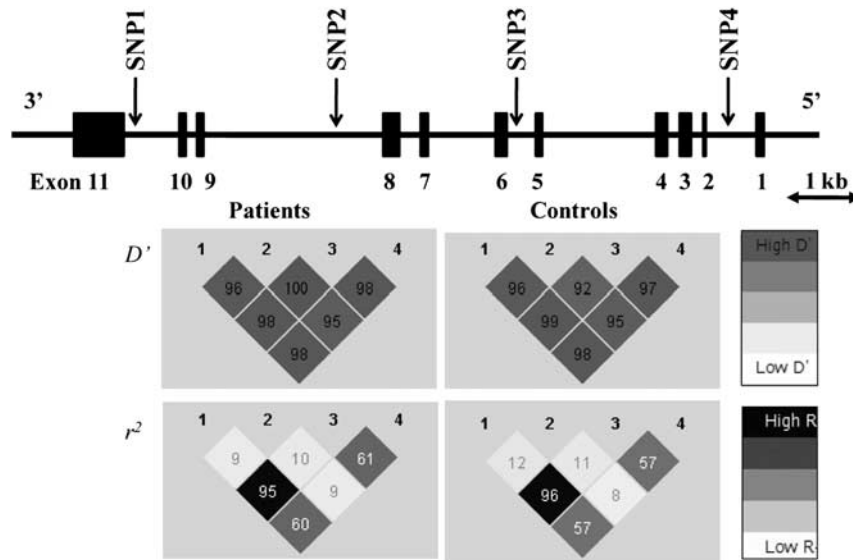
Figure 1 Genomic structure of *RELA*, including locations of the four SNPs studied and linkage disequilibrium of these four SNPs in the patient and control groups. The genomic structure of *RELA* is based on an entry in the Entrez Gene database (National Center for Biotechnology Information). The locations of the SNPs analyzed in this study are indicated with arrows. The distances of the exons–introns and intermarkers are drawn to scale. The linkage disequilibrium (LD) between pairwise SNPs, using D' and r^2 values, are shown at the bottom of the gene structure map separately for 1224 cases and 1663 controls. High levels of LD are represented by increasing gray scale intensity from 0 to 100, as shown by the bars.

Startle Response Measurement

A computerized human startle response monitoring system (Startle Eyeblink Reflex Analysis System Map1155SYS, NIHONSANTEKU, Osaka, Japan) was used to measure PPI. The methods for the startle paradigm, eyeblink acquisition, scoring parameters, and the procedure are described in detail elsewhere (Takahashi *et al*, 2008, 2010; Moriwaki *et al*, 2009). The startle paradigm was a total of 44 trials that consisted of three blocks with a continuous 70 dB sound pressure level (SPL) background white noise. Pulse stimuli consisted of broadband white noises at 115 dB SPL with an instantaneous rise/fall time of 40 ms. The pre-pulse stimuli were also broadband white noises with an instantaneous rise/fall time of 20 ms presented at three different intensities (82, 86, and 90 dB SPL). The lead interval (from pre-pulse onset to pulse onset) was 120 ms. In block 1, the startle response for the pulse alone trial (PA trial) was recorded six times. Block 2 consisted of PA trials or trials of pulse with pre-pulse at the three different intensities (PP trials) performed eight times for each condition. Block 3 was the same as block 1 to observe the habituation phenomenon. All trials were presented in a fixed pseudorandom order and were separated by inter-trial intervals of 15–25 s (20 s on average). The session lasted approximately 20 min, including 5 min of acclimation to the background noise. The following startle measures were calculated: (i) for the acoustic startle reflex, the average eyeblink amplitude of startle response to PA trials in block 1; (ii) habituation of the startle response during the session, calculated as the percentage of amplitude reduction between blocks 1 and 3 with the formula ((1−average eyeblink amplitude of the startle response in block 3/average eyeblink amplitude of the startle response in block 1) × 100); and (iii) PPI82, PPI86, PPI90, the pre-pulse inhibitions at

intensities of 82 dB, 86 dB, and 90 dB SPL, respectively. The PPI for each intensity level was calculated as the percentage of the amplitude reduction between the PA and PP trials in block 2 with the following formula: (1−average eyeblink amplitude of the startle response in the PP trials in block 2/average eyeblink amplitude of the startle response in the PA trials in block 2) × 100.

Statistical Analyses

Differences in clinical characteristics between patients and controls or between genotype groups were analyzed using the χ^2-test for categorical variables and the Mann–Whitney *U*-test for continuous variables using the PASW Statistics 18.0. software (SPSS Japan, Tokyo, Japan). Statistical analyses for genetic association were performed using the SNPAlyze v5.1.1 Pro software (DYNACOM, Yokohama, Japan). A logistic regression analysis (forced entry method) was conducted to examine the independent association of the sex (1: male, 2: female) and each genotype (0:M/M, 1:M/m, 2:m/m; M:major allele, m:minor allele) on the categorical diagnosis of schizophrenia (0: control, 1: patient). Sex and genotype statuses were included in the model as independent variables, and diagnosis was included as dependent variables. Deviation from the Hardy-Weinberg equilibrium (HWE) was tested separately in cases and controls using χ^2-tests for goodness of fit. The allelic and genotypic distributions of *RELA* polymorphisms between patients and controls were analyzed using χ^2-tests. 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). Haplotype frequencies were estimated by the maximum likelihood method using the genotyping data and the

expectation-maximization algorithm. Rare haplotypes found in less than 3% of both patients and controls were excluded from the association analysis. We performed 10 000 permutations for the most significant tests to determine the empirical significance. We used a 2–4-window fashion analysis. Pairwise linkage disequilibrium (LD) analyses, expressed by D' and r^2, were applied to detect the intermarker relationships in each group using the Haploview 4.1 software (http://www.broad.mit.edu/mpg/haploview/contact.php). We performed *post hoc* power calculations using the Power Calculator for Two Stage Association Studies (http://www.sph.umich.edu/csg/abecasis/CaTS (Skol *et al*, 2006)). Power estimates were based on the allele frequencies in patients, which ranged from 0.43 (SNP1) to 0.45 (SNP4); the odds ratios, which ranged from 1.16 (SNP1) to 1.23 (SNP4), for each associated SNP in this study and an alpha level of 0.05. Power was calculated under a prevalence of 0.01 using a multiplicative model, which assumed varying degrees of marker allele frequency and odds ratios. The effects of the *RELA* genotypes on the PPI in patients with schizophrenia were analyzed by one-way analysis of covariance (ANCOVA) to adjust for possible confounding factors (gender, current smoking status, and age) using the PASW Statistics 18.0 software (Swerdlow *et al*, 2008). Standardized effect sizes were calculated using Cohen's *d* method (http://www.uccs.edu/faculty/lbecker). All *p*-values reported are two tailed. Statistical significance was defined as $p < 0.05$.

RESULTS

Genetic Association Analysis

The genotype and allele frequencies of four SNPs located in the *RELA* gene are summarized in Table 1. The genotyping call rates were 96.4% (SNP1), 99.3% (SNP2), 96.7% (SNP3), and 97.5% (SNP4). No deviation from HWE was detected in the cases or controls ($p > 0.05$). Significant differences in the genotype and allele frequencies between patients and controls were observed for SNP1, SNP3, and SNP4 (SNP1: genotype $\chi^2 = 7.1$, $p = 0.028$, allele $\chi^2 = 7.0$, $p = 0.0080$; SNP3: genotype $\chi^2 = 8.6$, $p = 0.014$, allele $\chi^2 = 8.8$, $p = 0.0031$; SNP4: genotype $\chi^2 = 14.7$, $p = 0.00064$, allele

$\chi^2 = 14.9$, $p = 0.00011$). These associations remained significant even after the SNPSpD correction for multiple SNP tests, except for the genotypic association for SNP1 (the effective number of independent marker loci: 2.76: corrected *p* values, SNP1: genotype 0.078, allele 0.0021; SNP3: genotype 0.039, allele 0.0086; SNP4: genotype 0.0018, allele 0.00030). The frequencies of the C allele of SNP1, the G allele of SNP3 and the T allele of SNP4 were higher in patients than in controls. There was no allelic or genotypic association between SNP2 and schizophrenia. We additionally performed association analyses in males and females, separately. The association between the *RELA* gene and schizophrenia was found in males but not in females (Table 2). As the gender ratio was not matched in this sample, a logistic regression analysis (dependent variable: diagnosis, independent variables: genotype and gender) was performed for four SNPs. This analysis showed that the sex and each genotype were significant predictors for diagnosis of schizophrenia (all $p < 0.05$), except for genotype of SNP2 ($p = 0.19$) (Supplementary Table S1). Diagnosis of schizophrenia was significantly predicted by sex and genotype (SNP1, SNP3, and SNP4), respectively.

Two-four SNP sliding window haplotype analysis revealed significant association of this gene with schizophrenia (the lowest global $p = 0.00040$, SNP3-SNP4) (Table 3). The differences in the detailed haplotype frequencies between cases and controls are shown in Table 3 (lowest $p = 0.00006$, G-C haplotype compared with other haplotypes of SNP3-SNP4). The LD relationships between markers are provided in Figure 1. As the strong LD pattern observed in patients with schizophrenia was nearly identical to that among our controls and the JPT, CEU, and YRI HapMap samples, the strong LD patterns in this gene were likely to be common among ethnic groups.

In Silico Genotype-Expression Analysis

We analyzed the associations between the SNPs and the expression levels of the *RELA* gene in lymphoblasts in GeneVar database (Table 4). Unfortunately, data concerning SNP2 was not available in this database. We found that there were significant correlations between the *RELA* gene expression and all three SNPs associated with schizophrenia

Table 1 Genotypic and Allelic Distributions for SNPs in the *RELA* Gene Between Patients with Schizophrenia and Controls

Marker		M/m	Location	SCZ			CON			Genotypic *p* value (df = 2)	SCZ	CON	Allelic *p* value (df = 1)	OR (95%CI)
SNP ID	Position[a]			M/M	M/m	m/m	M/M	M/m	m/m		MAF			
SNP1	10728386	C/T	Intron 10	0.32	0.48	0.19	0.29	0.48	0.23	**0.028**	0.43	0.47	**0.008**	0.87 (0.78–0.96)
SNP2	10730962	G/C	Intron 8	0.78	0.21	0.0091	0.76	0.23	0.01	0.45	0.12	0.13	0.22	0.90 (0.77–1.06)
SNP3	10733141	G/A	Intron 5	0.32	0.48	0.20	0.28	0.48	0.24	**0.014**	0.44	0.48	**0.0031**	0.85 (0.77–0.95)
SNP4	10735731	C/T	Intron 1	0.31	0.48	0.21	0.36	0.47	0.16	**0.00064**	0.45	0.40	**0.00011**	1.23 (1.11–1.37)

Abbreviations: CI, confidence interval; CON, controls; M, major allele; m, minor allele; MAF, minor allele frequency; OR, odds ratio; SNP, single-nucleotide polymorphism; SCZ, patients with schizophrenia.
All the alleles are represented according to the minus strand DNA sequence.
P values < 0.05 are in bold and underlined.
[a] db SNP build 129.

Table 2 Genotypic and Allelic Distributions for SNPs in the *RELA* Gene Between Patients in Schizophrenia and Controls

(a) In males

Marker		M/m	Location	SCZ (N = 623)			CON (N = 773)			Genotypic *p* value (df = 2)	SCZ	CON	Allelic *p* value (df = 1)	OR (95%CI)
SNP ID	Position[a]			M/M	M/m	m/m	M/M	M/m	m/m		MAF			
SNP1	10728386	C/T	Intron 10	0.35	0.47	0.18	0.28	0.48	0.23	**0.014**	0.42	0.48	**0.0030**	0.79 (0.68–0.92)
SNP2	10730962	G/C	Intron 8	0.76	0.23	0.01	0.75	0.24	0.01	0.88	0.12	0.13	0.69	0.95 (0.76–1.20)
SNP3	10733141	G/A	Intron 5	0.34	0.47	0.19	0.28	0.48	0.24	**0.0080**	0.42	0.48	**0.0016**	0.78 (0.67–0.91)
SNP4	10735731	C/T	Intron 1	0.30	0.48	0.22	0.38	0.45	0.16	**0.0022**	0.46	0.39	**0.00037**	1.32 (1.13–1.54)

(b) In females

Marker		M/m	Location	SCZ (N = 601)			CON (N = 890)			Genotypic *p* value (df = 2)	SCZ	CON	Allelic *p* value (df = 1)	OR (95%CI)
SNP ID	Position[a]			M/M	M/m	m/m	M/M	M/m	m/m		MAF			
SNP1	10728386	C/T	Intron 10	0.30	0.50	0.20	0.30	0.48	0.23	0.52	0.45	0.47	0.45	0.94 (0.81–1.10)
SNP2	10730962	G/C	Intron 8	0.80	0.19	0.01	0.77	0.22	0.01	0.35	0.11	0.12	0.16	0.84 (0.67–1.07)
SNP3	10733141	G/A	Intron 5	0.30	0.49	0.21	0.29	0.47	0.24	0.52	0.45	0.47	0.32	0.93 (0.80–1.08)
SNP4	10735731	C/T	Intron 1	0.31	0.49	0.20	0.34	0.49	0.16	0.13	0.45	0.41	0.054	1.16 (1.00–1.35)

CI, confidence interval; CON, controls; M, major allele; m, minor allele; MAF, minor allele frequency; OR, odds ratio; SNP, single-nucleotide polymorphism; SCZ, patients with schizophrenia.
All the alleles are represented according to the minus strand DNA sequence.
P values <0.05 are in bold and underlined.
[a]db SNP build 129.

only in the Japanese population (SNP1: $r^2 = 0.27$, $p = 0.0003$; SNP3: $r^2 = 0.25$, $p = 0.0005$; SNP4: $r^2 = 0.15$, $p = 0.0089$). Interestingly, the risk alleles in all SNPs were associated with lower gene expression (SNP1: C allele, SNP3: G allele, SNP4: T allele).

Because SNP4 is located in intron 1, this SNP is possibly related to the regulation of gene transcription. An *in silico* search for potential transcription factor binding sites in the sequences surrounding SNP4 by the Patch 1.0 program showed that SNP4 could potentially alter androgen receptor (AR) binding. The consensus sequence of the AR binding site is AAAA<u>C</u>T (C allele of SNP4 is a non-risk allele). Thus, the mismatch in the AR consensus sequence created by the single nucleotide change of SNP4 (C allele to risk T allele: AAAA<u>T</u>T) could lead to lower transcriptional activity of the *RELA* gene, which is consistent with *in silico* expression data and genetic association results in male.

The Effect of *RELA* Genotypes on PPI

We examined possible associations between the genotype of four SNPs and PPI in patients with schizophrenia, as PPI is one of physiological phenotypes of schizophrenia. There was no difference in the demographic variables, including age, sex, current smoking status, years of education, chlorpromazine equivalents of total anti-psychotics, age at onset, duration of illness, medicated years with anti-psychotics, acoustic startle reflex, and habituation, between

genotype groups, except for the chlorpromazine equivalents of total anti-psychotics of SNP1 ($p = 0.028$) and SNP3 ($p = 0.043$) (Supplementary Table S2). One-way ANCOVA showed significant effects of the four genotypes on at least one of the PPI conditions (Table 5). The SNPSpD correction for multiple SNPs tested, revealed the association between three at risk genotypes (SNP1, SNP3, and SNP4) and PPI (SNP1 PPI86: corrected $p = 0.033$, PPI90: corrected $p = 0.044$; SNP3 PPI86: corrected $p = 0.036$, PPI90: corrected $p = 0.044$; SNP4 PPI82: corrected $p = 0.019$) (Table 5, Figure 2). However, the effects of the non-risk SNP (SNP2) on PPI was no longer significant after the SNPSpD correction (PPI86: corrected $p = 0.083$) (Table 5, Figure 2). The patients with the C/C at risk genotype of SNP1, patients with the G/G at risk genotype of SNP3 and patients with the T/T at risk genotype of SNP4 showed significant deficits in PPI.

DISCUSSION

In this study, we first provided evidence that genetic variants of the *RELA* gene are associated with the risk for schizophrenia. Next, *in silico* analysis suggested that the risk SNPs in the *RELA* gene might be associated with gene expression differences in lymphoblasts. Finally, we measured the effects of the *RELA* genotypes on PPIs in patients with schizophrenia. Our results indicated that the risk alleles were associated with reduced PPIs.

Table 3 Haplotype Analysis of the *RELA* Gene Between Patients and Controls

Haplotype[a]	Frequency		Individual *p* (χ^2)	Global *p* (χ^2)
	Patients	Controls		
SNP1-SNP2				**0.0019 (12.13)**
1–0	0.43	0.47	**0.012 (6.88)**	
0–0	0.45	0.40	**0.00060 (12.10)**	
0–1	0.12	0.13	0.19 (1.59)	
SNP2-SNP3				**0.00080 (13.24)**
0–1	0.44	0.48	**0.0053 (8.03)**	
0–0	0.45	0.40	**0.00030 (13.24)**	
1–0	0.12	0.13	0.23 (1.38)	
SNP3-SNP4				**0.00040 (15.14)**
1–0	0.43	0.48	**0.0031 (9.19)**	
0–1	0.45	0.40	**0.00006 (15.14)**	
0–0	0.12	0.13	0.21 (1.56)	
SNP1-SNP2-SNP3				**0.0022 (12.03)**
1–0–1	0.43	0.47	**0.0071 (7.07)**	
0–0–0	0.45	0.40	**0.00070 (12.02)**	
0–1–0	0.12	0.13	0.22 (1.40)	
SNP2-SNP3-SNP4				**0.00090 (13.66)**
0–1–0	0.44	0.48	**0.0028 (8.68)**	
0–0–1	0.45	0.40	**0.00040 (13.65)**	
1–0–0	0.11	0.12	0.26 (1.20)	
SNP1-SNP2-SNP3-SNP4				**0.0022 (13.11)**
1–0–1–0	0.43	0.47	**0.0058 (8.32)**	
0–0–0–1	0.45	0.40	**0.00060 (13.11)**	
0–1–0–0	0.12	0.12	0.27 (1.17)	

0, major allele; 1, minor allele. Haplotypes with frequencies of <3% in each group were excluded.
Significant *p* values <0.05 are represented by bold faces and underlines.

Table 4 Association Between the *RELA* Gene SNPs and mRNA Expression in a Japanese Population

SNP	Location	Population	*r*	Beta	SE	*t*	*p*
SNP1	Intron 10	JPT	0.52	0.24	0.06	3.91	**0.0003**
		CHB	−0.05	−0.02	0.06	−0.30	0.76
		CEU	−0.07	−0.03	0.05	−0.52	0.61
		YRI	0.15	0.05	0.05	1.16	0.25
SNP3	Intron 5	JPT	−0.50	−0.23	0.06	−3.74	**0.00050**
		CHB	0.05	0.02	0.06	0.30	0.76
		CEU	0.07	0.03	0.05	0.51	0.61
		YRI	−0.13	−0.05	0.05	−1.01	0.32
SNP4	Intron 1	JPT	−0.39	−0.17	0.06	−2.74	**0.0089**
		CHB	0.11	0.04	0.06	0.74	0.47
		CEU	−0.01	0.00	0.05	−0.06	0.95
		YRI	−0.16	−0.05	0.04	−1.20	0.24

JPT, Japanese in Tokyo, Japan; CHB, Han Chinese in Beijing, China; CEU, Utah residents with Northern and Western European ancestry from the CEPH collection (Parent); YRI, Yoruban in Ibadan, Nigeria (Parent).
P values <0.05 are in bold and underlined.

**Table 5** Association Between PPI and *RELA* Genotypes in Patients with Schizophrenia

SNP ID	PPI		*P* value (*F* value)	Corrected *P*	Cohen's *d*
SNP1	**C/C**	**T carrier**			
PPI82	17.0 ± 8.8	37.1 ± 4.4	**0.024 (5.39)**	0.066	−0.63
PPI86	14.1 ± 12.1	38.9 ± 4.6	**0.012 (6.75)**	**0.033**	−0.61
PPI90	24.2 ± 8.0	42.4 ± 4.8	**0.016 (6.25)**	**0.044**	−0.58
SNP2	**G/G**	**C carrier**			
PPI82	31.5 ± 5.1	28.1 ± 6.6	0.95 (<0.01)	>0.99	0.12
PPI86	38.0 ± 4.5	7.0 ± 15.1	**0.030 (4.97)**	0.083	0.73
PPI90	41.9 ± 4.8	18.2 ± 7.7	0.072 (3.38)	0.20	0.82
SNP3	**G/G**	**A carrier**			
PPI82	17.0 ± 8.8	36.5 ± 4.4	**0.029 (5.06)**	0.080	−0.62
PPI86	14.1 ± 12.1	39.3 ± 4.3	**0.013 (6.73)**	**0.036**	−0.64
PPI90	24.2 ± 8.0	42.7 ± 4.7	**0.016 (6.30)**	**0.044**	−0.61
SNP4	**T/T**	**C carrier**			
PPI82	8.6 ± 12.5	36.6 ± 4.0	**0.0068 (8.00)**	**0.019**	−0.81
PPI86	22.3 ± 10.2	32.9 ± 6.0	0.35 (0.89)	0.97	−0.29
PPI90	21.4 ± 11.5	40.2 ± 4.5	**0.041 (4.40)**	0.11	−0.56

SNP1: C/C, patients with C/C genotype ($n = 17$); T carrier, patients with C/T or T/T genotype ($n = 37$). SNP2: C carrier, patients with C/C or C/G genotype ($n = 12$); G/G, patients with G/G genotype ($n = 42$). SNP3: G/G, patients with G/G genotype ($n = 17$); A carrier, patients with G/A or A/A genotype ($n = 35$). SNP4: T/T, patients with the T/T genotype ($n = 11$); C carrier, patients with the T/C or C/C genotypes ($n = 42$). Means ± SE are shown. The effects of the *RELA* genotypes on PPI were analyzed by ANCOVA with age, sex, and current smoking status as covariates.
P values <0.05 are in bold face and underline. SNPSpD correction was applied to correct for multiple SNPs tested (the effective number of independent marker loci: 2.76).

As the association of the *RELA* gene and schizophrenia was supported by a number of statistical analyses such as genotypic and allelic associations for four SNPs (total 28) and haplotype analysis (total 24), the correction for multiple testing should be considered. In this study, overall genetic association tests were 52; however, all tests were not independent and multiple 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 only applied SNPSpD correction for genotypic and allelic association analysis for four SNPs, because the number of effective independent SNPs could be calculated by the SNPSpD method.

The reason why we obtained such low *p* values in our association analysis could be due to a relatively large sample size and high frequency of minor allele of the SNP4. Indeed, power analysis showed that our subjects had sufficient power (>0.95) to detect an effect of the odds ratios for SNP4, 1.23 in total subjects and 1.32 in male subjects. Although the strong association between the *RELA* gene and schizophrenia has been observed, the biological significance of this gene in susceptibility for schizophrenia might not be large, because 22% of the patients with schizophrenia have homozygous of risk allele in SNP4, but 16% of the controls also are homozygous of risk allele in SNP4. However, the association between the *RELA* gene and schizophrenia
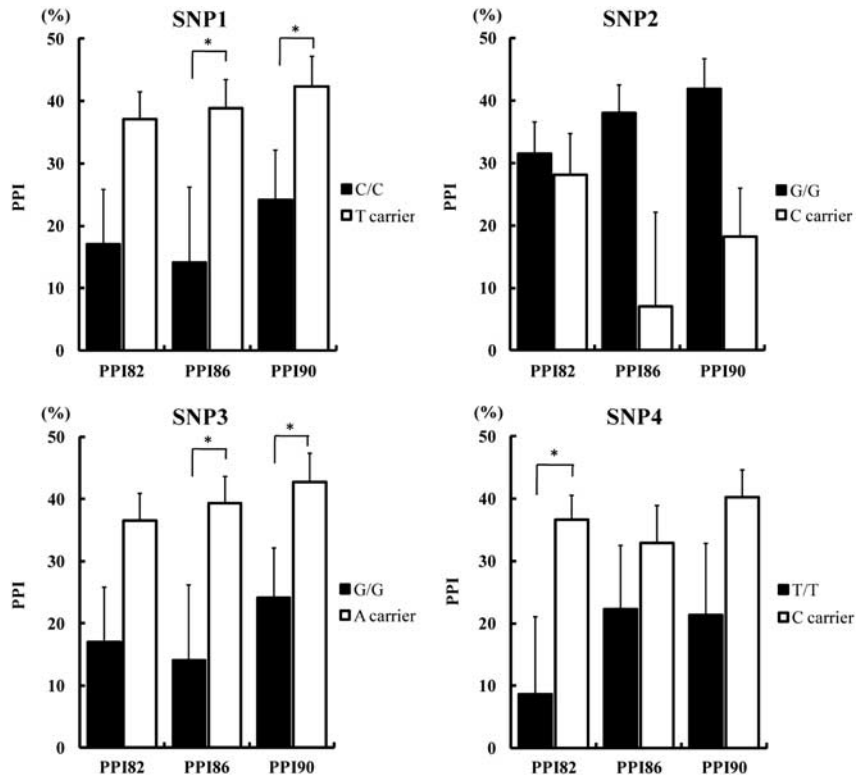
Figure 2 Effects of the genotypes in the RELA gene on PPI. PPI82, PPI86, and PPI90: pre-pulse inhibition of acoustic startle reflex in pre-pulse intensities of 82 dB, 86 dB, and 90 dB, respectively. Error bars represent the standard error of the mean. $*p < 0.05$. $**p < 0.01$.

might explain, at least in part, the relation between immune system and schizophrenia.

It is of interest to study how genetic variation affects *RELA* function/expression. There is no experimental-based evidence that any of the SNPs or haplotypes is functional. Very little is known about the potential function of specific intronic sequences with regard to protein binding, stability, and splicing efficacy. However, the genotype-expression analysis showed that the risk alleles are associated with lower *RELA* gene expression in Japanese lymphoblasts. On the other hand, no association was found between other SNPs and gene expression in other ethnicities. No data could explain these results. A possible explanation for this ethnic difference is that the allele frequencies of some target genes in the signal transduction pathway of NF-κB, such as interleukins, chemokines, and interferons, and molecules involved in apoptosis or adhesion (Li and Verma, 2002), which could be associated with the pathophysiology of schizophrenia, might be different among populations. For example, a GWAS study in Caucasian population showed significant association with schizophrenia were in a region of LD on chromosome 6p22.1, including several immunity related genes other than the *RELA* gene (Stefansson *et al*, 2009). There were four SNPs associated with schizophrenia in the immunity related genes. Three SNPs out of four SNPs were not polymorphic in Japanese population and the minor allele frequency of the remained one SNP is 0.01 in Japanese population (0.19 in Caucasian population). This difference of allele frequency of SNP could alter the effect of the presumably functional risk

alleles in the *RELA* gene in other ethnicities. A risk SNP of the *RELA* gene was found in the possible transcription factor binding sequence of AR. Indeed, it was reported that AR activation decreased the expression of *RELA* and reduced its nuclear localization and transcriptional activity (Nelius *et al*, 2007). As the risk allele destroyed the consensus sequence for AR binding, this SNP could be functional. Several studies have examined the association between AR and schizophrenia, and there are clinical differences between males and females, such as greater lifetime risk, earlier age of onset and poorer outcome in males (Tandon *et al*, 2008). Crow *et al*. reported the association of AR with schizophrenia in males (Crow *et al*, 1993); however, negative results have also been reported (Arranz *et al*, 1995; Tsai *et al*, 2006). When we examined the association between the *RELA* gene and schizophrenia by gender, a significant association was observed in males but not in females. Our results suggest that the T allele of SNP4 in the *RELA* gene, which might be functional for AR binding and transcription, could be a risk-associated allele for male schizophrenia.

There are numerous genes in neurons that are regulated by NF-κB (Kaltschmidt *et al*, 2005). Among these genes are molecules related to neurotransmission, including subunits of N-methyl-D-aspartate receptors, voltage-dependent calcium channels and the calcium-binding protein calbindin; cell survival factors, including Bcl-2, Mn-SOD, and inhibitor of apoptosis proteins (IAPs); and cell death factors, including Bcl-x(S) and Bax (Mattson, 2005). It is noteworthy that the expression levels of some of these downstream

molecules have been reported to be altered in postmortem brains of patients with schizophrenia, for example, increased: Bax, calbindin, and NR2B; decreased Bcl-2 (Gao *et al*, 2000; Jarskog *et al*, 2000, 2004; Fung *et al*, 2010).

PPI of the acoustic startle response has been demonstrated from mice to humans, and is considered to be a measure of 'sensorimotor gating,' whereby pre-pulses reduce the effect of subsequent sensory stimuli to protect the brain from sensory overload (Braff and Geyer, 1990). A deficit in PPI is a reliable feature of schizophrenia, where reduced gating is thought to be one possible neurobiological mechanism that underlies the basic cognitive abnormalities associated with this disorder (Braff *et al*, 2001). We observed associations between risk SNPs in the *RELA* gene and some pre-pulse intensities in patients with schizophrenia. Although we cannot explain the differences in the associated pre-pulse intensities among SNPs, our findings suggest that the *RELA* gene might modulate PPI in patients with schizophrenia.

The PPI deficits observed in schizophrenia can be mimicked in animals by the administration of dopamine agonizts and NMDA antagonists, such as phencyclidine (PCP), and reversed by anti-psychotic drugs (Mansbach *et al*, 1988; Geyer *et al*, 2001; Wang *et al*, 2001). Typical and atypical anti-psychotic drugs also reverse the PPI deficits observed in schizophrenic patients (Kumari and Sharma, 2002). Consistent with our data, PCP administration to rats that showed deficits in PPI elicited the abnormal nuclear translocation of NF-κB in the frontal cortex (Wang *et al*, 2001), which is indicative of a functional correlation between the *RELA* gene and PPI in a potential animal model for schizophrenia.

There are several limitations to interpret our results. Our study size of 1224 cases and 1663 controls had sufficient power (>0.80) to detect the effects of odds ratios of 1.16 or greater as indicated in recent genome-wide association studies for each SNP (O'Donovan *et al*, 2009). However, the possibility of false positive results due to type I errors could not be excluded. Our positive results might be derived from sample bias due to population stratification and non-sex-matched samples, although the Japanese are a relatively homogeneous population and the logistic regression study revealed genotype effects on the diagnosis independent of sex. We did not perform a systematic mutation search using these Japanese schizophrenia samples. *In silico* analysis of gene expression in lymphoblasts and AR binding site in SNP4, raised a possibility that the SNP4 might be a functional SNP. However, further biological study of the function of SNP4 is required to verify these *in silico* results. As the sample size we used for the PPI analysis was small, gender effect on the association between PPI and the SNPs in the *RELA* gene was not able to be analyzed. An increased sample size for schizophrenia and control subjects is needed before a firm conclusion can be drawn. We used PPI as a main phenotype of interest. Other phenotypes such as neurocognitive dysfunction that has larger effect size than PPI and brain morphology, which is more stable overtime were not tested in this study; however, PPI is a physiological phenotype, which is reliable, easily measured, and relatively specific for schizophrenia. In the present study, we propose *RELA* as a new candidate gene for susceptibility to schizophrenia.

DISCLOSURE

The authors RH, KO, YY, MF, HY, HT, MI, TO, HK, OS, MT, NI, NO, KK, HK and MT declare that no financial support or compensation has been received from any individual or corporate entity over the past 3 years for research or professional service and there are no personal financial holdings that could be perceived as constituting a potential conflict of interest.

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