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SA4503, a sigma-1 receptor agonist, prevents cultured cortical neurons from oxidative stress-induced cell death via suppression of MAPK pathway activation and glutamate receptor expression

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ABSTRACT

Many studies suggest that antidepressants act as neuroprotective agents in the central nervous system (CNS), though the underlying mechanism has not been fully elucidated. In the present study, we examined the effect of SA4503, which is a sigma-1 receptor agonist and a novel antidepressant candidate, on oxidative stress-induced cell death in cultured cortical neurons. Exposure of the neurons to H_2O_2 induced cell death, while pretreatment with SA4503 inhibited neuronal cell death. The SA4503-dependent survival effect was reversed by co-application with BD1047 (an antagonist of sigma-1/2 receptors). Previously we found that H_2O_2 triggers a series of events including over-activation of mitogen-activated protein kinase/extracellular signal-regulated kinase (MAPK/ERK) and intracellular Ca^{2+} accumulation via voltage-gated Ca^{2+} channels and ionotropic glutamate receptors, resulting in neuronal cell death (Numakawa et al. (2007) [20]). Importantly, we found in this study that SA4503 reduced the activation of the MAPK/ERK pathway and down-regulated the ionotropic glutamate receptor, GluR1. Taking these findings together, it is possible that SA4503 blocks neuronal cell death via repressing activation of the MAPK/ERK pathway and, consequently, expression levels of glutamate receptors.

The sigma-1 receptor (sig-1R) is a possible target to treat for several brain-related illnesses [6], because sig-1R is putatively involved in synaptic plasticity and neuroprotection in the CNS. Sig-1R has been shown to play a role in critical intracellular processes that regulate Ca^{2+} signaling and protein transport. Hayashi and Su demonstrated that the endoplasmic reticulum (ER) protein Sig-1R is a Ca^{2+} (via inositol 1,4,5-trisphosphate receptors, IP_3R)-sensitive and ligand-operated receptor chaperone at the ER membrane [7]. Sig-1R chaperones at the ER-mitochondrion interface regulate Ca^{2+} signaling and cell survival.

SA4503 [1-(3,4-dimethoxyphenethyl)-4-(3-phenylpropyl) piperazine dihydrochloride] is recognized as a selective ligand for sig-1R chaperones [15]. Previous studies suggest that SA4503 positively affects memory by improving memory impairments and increasing cell survival [25,26]. Up-regulation of hippocampal BDNF levels (brain-derived neurotrophic factor), which play a crucial role in synaptic function and neuronal survival [11,21,29,30], have been demonstrated after chronic treatment with SA4503 [8]. Previously, we reported that BDNF rapidly induces release of the neurotransmitter glutamate through PLCγ/IP_3R/Ca^{2+} signaling and that antidepressants, including imipramine and fluvoxamine, enhance the PLCγ/IP_3R/Ca^{2+} system via Sig-1R [19,32]. These studies indicate that Sig-1R has multiple roles in the CNS.

Interestingly, SA4503 shows antidepressant-like, and neuroprotective effects [16,27]. SA4503 decreased the immobility time in the forced swim test in rats, suggesting that SA4503 may have potential antidepressive properties [27]. Moreover, in the same study, SA4503 showed a synergistic effect with imipramine during the forced swim test. Lucas et al. reported further evidence supporting the antidepressant potential of SA 4503 through electrophysiological, morphological and behavioural studies [12]. In addition, Nakazawa et al. showed that SA4503 demonstrates a protective effect on neuronal cultures against the hypoxia/hypoglycemia-induced neurotoxicity [16]. Thus, it is possible that SA4503 is a potential drug for treatment for several brain-related illnesses, including depressive disorder.

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However, little is known concerning detailed cellular mechanisms underlying the effect of SA4503 in CNS neurons.

Oxidative stress may be associated with various neurodegenerative diseases including Alzheimer's disease, Parkinson's disease, Huntington's disease and amyotrophic lateral sclerosis [1,2]. In this study, we examined whether SA4503 demonstrates a preventive effect on cultured cortical neurons from oxidative stress when SA4503 is applied before H_2O_2 exposure.

Primary cortical cultures were prepared from postnatal 2-day-old rats as previously reported [22,23]. The culture medium consisted of 5% fetal bovine serum, 5% heated-inactivated horse serum, 90% of a 1:1 mixture of Dulbecco's modified Eagle's medium, and Ham's F-12 medium. Dissociated cortical neurons were cultured for 4 or 5 days before SA4503 (a gift from M's Science Corporation, Hyogo, Japan) was applied. Twenty-four hours after SA4503 addition, H_2O_2 (final 50 μM) was applied for 12 h. Then, the cell viability was analyzed. To determine the cell viability, we carried out a mitochondrial-dependent conversion of the tetrazolium salt (MTT) assay. The metabolic activity of mitochondria was estimated with the MTT assay as previously reported [20]. BD1047 (1 μM, Tocris Cookson Ltd., Avonmouth, UK), an antagonist of sigma-1/2 receptors [13], was applied 20 min before adding SA4503. U0126 (an inhibitor for MEK, an upstream molecule of MAPK/ERK) was purchased from Promega (WI, USA), and used at a final concentration of 10 μM. 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX) was purchased from Tocris Bioscience (Bristol, UK). Other reagents were obtained from SIGMA (MO, USA). All animals were treated according to the institutional guidelines for the care and use of animals.

Immunostaining was carried out as reported previously with some modifications [18]. First, cultured cells were fixed in 4% paraformaldehyde at room temperature for 20 min. After three times washes with PBS, the cells were permeabilized, and the non-specific binding of antibodies was blocked with 10% goat serum, 0.2% Triton X-100 in PBS for 30 min at room temperature. The anti-MAP2 (1:1000, SIGMA) antibody was applied overnight at 4 °C. Following three times washes with PBS, Alexa Fluor 488-conjugated anti-mouse IgG (1:200, Invitrogen) was applied as a secondary antibody. Immunoreactivity was monitored with a fluorescence microscope (Axiovert 200, ZEISS, Tokyo, Japan).

Cells were lysed in SDS lysis buffer containing 1% SDS, 20 mM Tris–HCl (pH 7.4), 5 mM EDTA (pH 8.0), 10 mM NaF, 2 mM Na_3VO_4, 0.5 mM phenylarsine oxide, and 1 mM phenylmethylsulfonyl fluoride. The protein concentration was quantified using a BCA Protein Assay Kit (PIERCE), and equivalent amounts of total protein were assayed for each immunoblotting. Primary antibodies were used at the following dilutions: anti-Akt (1:1000, Cell Signaling, MA, USA), anti-pAkt (1:1000, Cell Signaling), anti-ERK (1:1000, Cell Signaling), anti-pERK (1:1000, Cell Signaling), anti-pJNK (1:1000, Cell Signaling), anti-JNK (1:1000, Cell Signaling), anti-β-actin (1:5000,

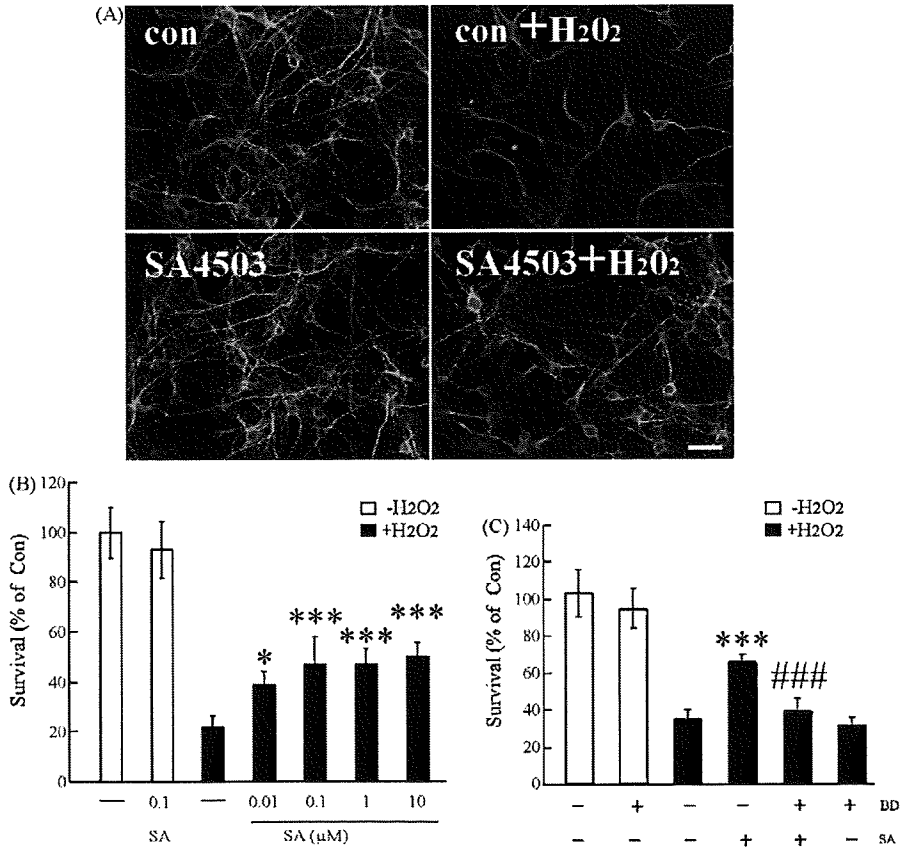

Fig. 1. SA4503 prevents cultured cortical neurons from cell death caused by H_2O_2 application. (A) SA4503 pretreatment inhibited the H_2O_2-induced neuronal cell death. SA4503, a sig-1R agonist, was applied to DIV4 cultures at 0.1 μM. Twenty-four hours later, H_2O_2 (final 50 μM) was added to induce cell death. After 12 h, cell survival was determined by immunostaining with anti-MAP2 antibody. Bar = 50 μm. (B) SA4503 exerted survival effects in a dose-dependent manner. SA4503 pretreatment (24 h) was performed at 0.01, 0.1 1, or 10 μM. The cultures were then incubated with H_2O_2 for 12 h, followed by MTT assay. Data represent mean ± SD ($n = 5$, n indicates the number of wells of a plate for each experimental condition). ***$P < 0.001$, *$P < 0.05$ vs. 0 μM SA4503 + H_2O_2 (one-way ANOVA). SA: SA4503. (C) BD1047, an antagonist of sigma-1/2 receptors, canceled the SA4503-dependent cell survival. BD1047 (1 μM) was applied 20 min before adding SA4503 (0.1 μM). MTT assay. Data represent mean ± SD ($n = 5$, n indicates the number of wells of a plate for each experimental condition). ***$P < 0.001$, vs. 0 μM SA4503 + H_2O_2. ###$P < 0.001$ vs. SA4503 + H_2O_2 (one-way ANOVA). BD: BD1047.

SIGMA), anti-NR2A (1:500, SIGMA), anti-NR2B (1:500, SIGMA), anti-GluR1 (1:1000, CHEMICON, CA, USA), and anti-GluR2/3 (1:500, CHEMICON) antibodies. The intensity of the immunoreactivity was quantified by using Lane & Spot Analyzer software (ATTO Corporation, Tokyo, Japan). The *n* indicates the number of experiments in separated cultures.

Data shown in this study are presented as mean ± standard deviation (SD). Statistical significance was evaluated using a one-way

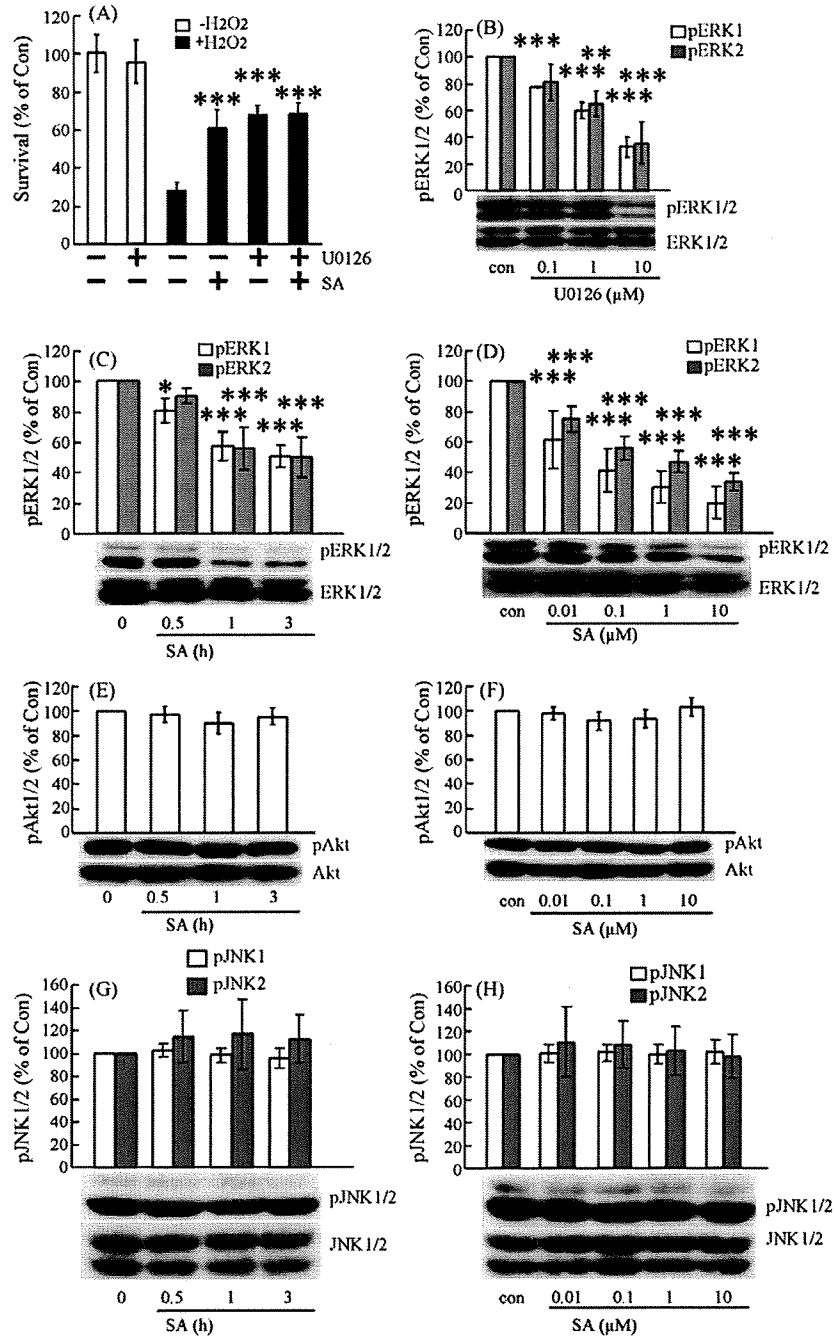

Fig. 2. The MAPK pathway is involved in H_2O_2-induced cell death and reduction in activation of MAPK is important for the protective effect of SA4503. (A) U0126 (a specific inhibitor of MEK, an upstream of MAPK) and SA4503 exerted a survival effect. U0126 (10 μM) was applied 3 h before exposure to H_2O_2. SA4503 (0.1 μM) was pretreated for 24 h. MTT assay. Data represent mean ± SD ($n = 6$). ***$P < 0.001$ vs. 0 μM SA4503 + H_2O_2 (one-way ANOVA). Co-application of U0126 with SA4503 did not show any additional or synergistic effects as compared with solo drug addition. (B) Inhibitory effect of U0126 on activation of MAPK/ERK. The levels of pERK1/2 (phosphorylated ERK1/2) were quantified (Western blotting). Data represent mean ± SD ($n = 4$). ***$P < 0.001$, **$P < 0.01$ vs. 0 μM U0126. Normalization to a level of pERK1/2 in control (without U0126) was performed. (C) SA4503 reduced activation of ERK1/2. SA4503 (0.1 μM) was applied for 0.5–3 h. The levels of pERK1/2 were quantified. Data represent mean ± SD (n = 4). Normalization to a level of pERK1/2 in control (without SA4503) was performed. ***$P < 0.001$, *$P < 0.05$ vs. 0 μM SA4503 (one-way ANOVA). (D) SA4503 reduced the pERK1/2 levels in a dose-dependent manner. SA4503 (0.01. 0.1, 1, or 10 μM) was applied for 3 h. Data represent mean ± SD ($n = 6$). ***$P < 0.001$ vs. 0 μM SA4503 (one-way ANOVA). The (E) time- or (F) dose-dependency of SA4503 on phosphorylated Akt (pAkt) was examined. SA4503 did not induce any change in the Akt activation. Data represent mean ± SD ($n = 4$). The (G) time- or (H) dose-dependency of SA4503 on phosphorylation of JNK1/2 (pJNK1/2). SA4503 did not have any effect. Data represent mean ± SD ($n = 6$).

ANOVA followed by Tukey's test in SPSS ver11 (SPSS Japan, Tokyo, Japan). Probability values less than 5% were considered statistically significant.

We first examined the effect of pretreatment with SA4503 on cell death caused by H_2O_2 application. SA4503 (0.1 μM) was applied to cultured cortical neurons at 4 days in vitro (DIV4). Twenty-four hours later, exposure to H_2O_2 (50 μM) was carried out to induce cell death. Following H_2O_2 treatment for 12 h, an immunostaining with anti-MAP2 (microtubule-associated protein 2, neuronal marker) antibody was performed. As shown in Fig. 1A, H_2O_2 induced significant neuronal cell death compared with the control (without SA4503 and H_2O_2). We found that SA4503 significantly reduced the cell death caused by H_2O_2 (Fig. 1A). We previously determined that the ratio of MAP2-positive cells to the total cells at DIV5 was about 80% in our cultures [14]. To further investigate cell survival, the MTT assay was also conducted. As illustrated, SA4503 decreased cell death by H_2O_2 at any dose of SA4503 (0.01–10 μM, Fig. 1B). The survival effect by SA4503 reached a plateau at 0.1 μM (Fig. 1B). Therefore, the following experiments for analyzing cell survival were performed with 0.1 μM of SA4503. Next, we examined the possible involvement of sigma-1/2 receptors in the SA4503 effect. Co-pretreatment with SA4503 and BD1047 (1 μM, an antagonist of sigma-1/2 receptors) failed to protect cortical neurons from cell death by H_2O_2 (Fig. 1C), suggesting that SA4503 has survival-promoting effect via sigma-1/2 receptors.

To clarify the mechanisms underlying the SA4503-dependent survival, we examined the change in activation of intracellular signaling. PI3K, phosphatidylinositol 3-kinase, and MAPK/ERK pathways are essential for neuronal survival in the CNS [5,24]. On the other hand, we previously reported that over-activation of ERK1/2 caused by H_2O_2 is involved in cell death [20]. Thus, to examine the activation of MAPK/ERK pathway to the downstream effects of SA4503, we applied an inhibitor of the MAPK/ERK pathway, U0126, to the cortical cultures. U0126 (10 μM, 3 h before H_2O_2 stimulation) significantly inhibited H_2O_2-dependent cell death (Fig. 2A), suggesting that the MAPK/ERK pathway is involved in cell death by H_2O_2. No additional or synergistic effect by co-application of U0126 and SA4503 as compared with solo SA4503 or U0126 application was observed (Fig. 2A), suggesting that reduction in the activation of the MAPK/ERK pathway contributes to cell protection by SA4503. We confirmed that a decrease in activation (phosphorylation) of p44/42 MAPK (ERK1/2) after U0126 exposure (3 h) occurred in a dose-dependent manner, though the total expression of ERK1/2 was not changed (Fig. 2B). Next, we examined activation of ERK1/2 after SA4503 exposure. SA4503 treatment for 0.5–3 h reduced levels of pERK1/2 (Fig. 2C). The total ERK1/2 was not changed by SA4503 (Fig. 2C). SA4503 depressed the pERK1/2 levels in a dose-dependent manner (Fig. 2D). We checked the activation of Akt (a component of the PI3K pathway). When the time- or dose-dependency of SA4503 on Akt activation was determined, the activation levels were not influenced by SA4503 (Fig. 2E and F). Total Akt expression was intact after SA4503 application (Fig. 2E and F). Furthermore, both total JNK1/2 (c-JunNH2-terminal kinase1/2, an another member of MAPKs) and pJNK1/2, which regulate apoptosis [9,31], were examined. After the time- or dose-dependency of SA4503 was determined, it was revealed that total JNK1/2 and pJNK1/2 were not changed by SA4503 (Fig. 2G and H). These results suggest that SA4503 has a protective effect on cortical neurons via repressing activation of the MAPK/ERK pathway.

We investigated whether ionotropic glutamate receptors are involved in SA4503-dependent neuroprotection as contribution of glutamate function to oxidative stress was reported [17]. Interestingly, SA4503 dramatically decreased expression of GluR1 in a dose-dependent manner (Fig. 3A). Small decreases in the NR2A (not NR2B), and GluR2/3 expression levels after SA4503 exposure were also observed (Fig. 3A). The level of β-actin is shown as a control (Fig. 3A). The time-course analysis of SA4503-induced reduction of GluR1 expression was performed. Cultures were treated with SA4503 for 0.5–3 h, and down-regulation of GluR1 expression occurred at all time points (Fig. 3B). β-actin levels were intact after SA4503 application (Fig. 3B).

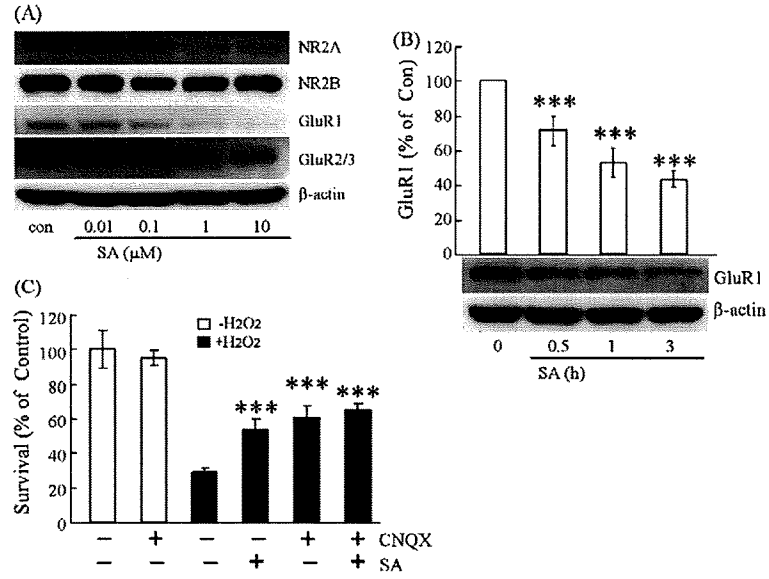

Fig. 3. SA4503 treatment down-regulates the levels of ionotropic glutamate receptors. (A) Expressions of glutamate receptor subunits including NR2A, NR2B, GluR1 and GluR2/3, were examined after SA4503 exposure. SA4503 (3 h) was applied at the indicated concentration. SA4503 decreased expression of GluR1 significantly. The small decreasing tendency in the NR2A and GluR2/3 expression levels was also observed. β-actin is shown as a control. (B) The time-course analysis of SA4503-induced reduction of GluR1 expression. After immunoblotting was performed, quantification was carried out. Normalization to a level in control (no SA4503 application) was performed. Data represent mean ± SD ($n = 5$). ***$P < 0.001$ (one-way ANOVA). β-actin is a control. (C) CNQX (AMPA glutamate receptors antagonist) blocked H_2O_2-induced death. CNQX (10 μM) was applied 20 min before H_2O_2 exposure. SA4503 (0.1 μM) was pretreated for 24 h. MTT assay. Data represent mean ± SD ($n = 5$). ***$P < 0.001$ vs. 0 μM SA4503 + H_2O_2 (one-way ANOVA). Neither of these drugs exerted any additional or synergistic effect as compared with solo treatment.

Furthermore, to elucidate the possibility that a change in the level of ionotropic glutamate receptors, such as GluR1 AMPA receptors, is associated with SA4503-mediated survival promotion, we tested the effect of CNQX, an antagonist for AMPA receptors. CNQX application (20 min) prevented cortical neurons from cell death by H_2O_2. As shown, no additional or synergistic effect of co-application of CNQX and SA4503 as compared with solo drug treatment was confirmed (Fig. 3C), implying that a decrease in AMPA receptor activation is involved in the SA4503-dependent survival against H_2O_2-mediated cell death.

In the present study, we found that SA4503, a sig-1R agonist and a novel antidepressant candidate, blocked H_2O_2-induced neuronal cell death. SA4503 caused the down-regulation of the MAPK/ERK pathway activation and reduced levels of ionotropic glutamate receptors. U0126, an inhibitor of the MAPK/ERK pathway, and CNQX, an inhibitor of AMPA glutamate receptors, prevented cultured cells from the H_2O_2-induced death. Remarkably, after co-treatment with U0126 or CNQX, SA4503 exerted no additional or synergistic survival effects as compared with solo application.

ERK1/2 (p44/p42MAPK) are two isoforms of ERK that belong to the family of MAPKs, including the JNK1/2 and the p38 MAP kinase. ERK activation controls various cell responses, including proliferation, survival, and synaptic maturation; indeed, we previously showed that neurotrophin BDNF up-regulated synaptic proteins via the MAPK/ERK pathway [10,14]. Paradoxically, depending on the duration, the magnitude and its subcellular localization, aberrant ERK activation can promote cell death [3]. Persistent activation of ERK contributes to glutamate-induced oxidative toxicity in cortical neurons [28]. Consistently, we previously found that H_2O_2 induced over-activation of ERK1/2 [20]. Crossthwaite et al. showed MAPK activation in cultured cortical neurons exposed to H_2O_2 (300 μM), however, U0126 did not rescue but slightly enhanced the cell death [4]. In our system, U0126 significantly inhibited H_2O_2-induced cell death, and marked reduction of activated ERK1/2 levels after SA4503 exposure was observed. In contrast, phosphorylation of JNK1/2, which is known as pro-apoptotic molecule [9,31], was not changed by SA4503 exposure. In addition, no additional or synergistic effect of co-application of U0126 and SA4503 on neuroprotection as compared with solo drug application was confirmed. These results suggest that SA4503 exerts the survival effect via repressing activation of the MAPK/ERK pathway. Importantly, though the reduction in activation of ERK1/2 induced by SA4503 or by U0126 was significant, a complete abolishment of basal activated ERK1/2 was not achieved by each drug treatment. Activation of MAPK/ERK signaling beyond the normal threshold may be toxic to cells, while basal activity of this signaling is essential for neuronal survival. Furthermore, there is a possibility that subcellular localization of pERK1/2 is involved in multiple functions of this kinase [3]. It may be valuable to study whether an unknown phosphatase that prevents the activation of ERK1/2 or upstream molecules of ERK1/2, is involved in SA4503-dependent inhibition of pERK1/2.

We previously reported that exposure to H_2O_2 causes a series of events including ERK1/2 over-activation and an increase in intracellular Ca^{2+} via voltage-gated Ca^{2+} channels and ionotropic glutamate receptors, ultimately resulting in cell death [20]. Under H_2O_2 stress, the ERK1/2 signal may work as a death mediator, as U0126 blocks cell death. We recently found that the MAPK/ERK pathway is also involved in maintenance of the expression of ionotropic glutamate receptors [10]. Indeed, in the present study, SA4503 induced the marked down-regulation of glutamate receptors (especially, GluR1). Therefore, we investigated the possibility that a decrease in AMPA receptor activation is required for the SA4503-dependent survival. As expected, CNQX (AMPA receptors antagonist) blocked H_2O_2-induced death, and any additional or synergistic effect of co-application of CNQX and SA4503 was not observed. It is possible that SA4503 protects neurons from oxidative toxicity via decreasing activation of ERK1/2, which is a critical signaling component for maintenance of GluR1 expression.

In summary, SA4503, a sig-1R agonist, stimulates survival-promoting effects on cultured cortical neurons. Previously, we found that antidepressants (imipramine, and fluvoxamine) potentiate BDNF-induced intracellular signaling for release of glutamate via stimulation of sig-1R [32]. Recently, up-regulation of BDNF protein in the rat hippocampus by chronic treatment with SA4503 has been reported [8]. Collectively, these results, including our present study, suggest that SA4503 plays various functions in the CNS. In addition to the potential as a novel antidepressant agent, SA4503 may be valuable to study as a therapeutic agent in the treatment of neurodegenerative diseases of the CNS, although further studies concerning intracellular mechanisms are needed.

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RESEARCH ARTICLE

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Association Study of Bromodomain-Containing 1 Gene With Schizophrenia in Japanese Population

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Chromosome 22q13 region has been implicated in schizophrenia in several linkage studies. Genes within this locus are therefore promising genetic and biologic candidate genes for schizophrenia if they are expressed in the brain or predicted to have some role in brain development. A recent study reported that bromodomain-containing 1 gene (*BRD1*), located in 22q13, showed an association with schizophrenia in a Scottish population. Except for being a putative regulator of transcription, the precise function of *BRD1* is not clear; however, expression analysis of *BRD1* mRNA revealed widespread expression in mammalian brains. In our study, we explored the association of *BRD1* with schizophrenia in a Japanese population (626 cases and 770 controls). In this association analysis, we first examined 10 directly genotyped single-nucleotide polymorphisms (SNPs) and 20 imputed SNPs. Second, we compared the *BRD1* mRNA levels between cases and controls using lymphoblastoid cell lines (LCLs) derived from 29 cases and 30 controls. Although the SNP (rs138880) that previously has been associated with schizophrenia showed the same trend in the Japanese population, no significant association was detected between *BRD1* and schizophrenia in our study. Similarly, no significant differences in *BRD1* mRNA levels in LCLs were detected. Taken together, we could not strongly show that common SNPs in the *BRD1* gene account for a substantial proportion of the genetic risk for schizophrenia in the Japanese population. © 2009 Wiley-Liss, Inc.

Key words: association analysis; imputation; gene expression analysis; meta-analysis

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INTRODUCTION

Schizophrenia is a severe, debilitating disorder characterized by delusional beliefs, hallucinations, disordered speech, and deficits in emotional and social behavior. It is strongly familial, and heritability is around 80% based on twin studies [Sullivan et al., 2003]. However, the pattern of inheritance is complex, with most studies suggesting an interaction of multiple genes. There are now several positional candidate regions all over the genome that have been shown to be related to schizophrenia in genetic studies [Badner and Gershon, 2002; Williams et al., 2003].

One promising region is chromosome 22q. Initial evidence for linkage to chromosome 22q came from three markers spanning ~23 cM in the 22q13.1 region in the Maryland family sample [Pulver et al., 1994]. Additional interest in 22q13 came from a genome scan of catatonic schizophrenia pedigrees, which showed suggestive evidence for linkage ($P = 1.8 \times 10^{-3}$; non-parametric logarithms of the odds [LOD] score 1.85) on 22q13 [Stober et al., 2000]. Furthermore, a multicenter linkage study that evaluated 10 microsatellite markers spanning 22q in 779 schizophrenia pedigrees

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showed linkage of borderline significance to D22S1169 at 22q13.32 in the total sample when intersample heterogeneity was taken into account [Mowry et al., 2004].

A recent study [Severinsen et al., 2006] looked into this 22q13 region and reported that two single-nucleotide polymorphisms (SNPs) (rs4468 and rs138880) located within bromodomain-containing 1 gene (*BRD1*) were associated with schizophrenia in a single-marker association analysis. This gene, expressed in mammalian brain tissue, encodes a protein of unknown function that contains a bromodomain, a motif often found in transcriptional coactivators. The motif represents an evolutionarily conserved nucleotide sequence found in many chromatin-associated proteins and in nearly all known nuclear histone acetyltransferases. It is therefore thought that *BRD1* is related to transcriptional regulation [Zeng and Zhou, 2002].

BRD1 is an attractive candidate gene for schizophrenia for two reasons. First, *BRD1* as a putative transcriptional cofactor might have functional implications for susceptibility to schizophrenia. Second, it also maps to the 22q13.33 locus, the region with evidence for linkage to schizophrenia.

As mentioned, a single study has implicated genetic variants within *BRD1* locus as contributing factor to schizophrenia in a Scottish population [Severinsen et al., 2006]. To further investigate this possible association, we selected SNPs within the *BRD1* locus and carried out a case–control study in a Japanese population. In terms of understanding the relationship between *BRD1* and schizophrenia, our study brings additional information from a genetic point of view: a larger sample size, a different population, and better coverage (in terms of SNPs selected for analysis).

MATERIALS AND METHODS

Subjects

All subjects were of Japanese descent and recruited from the main island of Japan. For the association analysis, 626 patients with schizophrenia and 770 healthy controls were used (Supplementary Table I). For the expression analysis, 29 patients with schizophrenia and 30 healthy controls were used (Supplementary Table II). All patients were diagnosed according to the Diagnostic and Statistical Manual of Mental Disorders, Fourth Edition, criteria based on the consensus of at least two experienced psychiatrists using an unstructured interview and review of medical records. All healthy controls were psychiatrically screened using an unstructured interview to exclude subjects with any brain disorder or psychotic disorder or who had first-degree relatives with psychotic disorders. The present study was approved by the Ethics Committees of Nagoya University, Fujita Health University. All subjects provided written informed consent after the study was described to them.

Tagging SNP Selection, SNP Genotyping, and Quality Control

The International Haplotype Mapping (HapMap) (www.hapmap.org) SNP database and ABI (Applied Biosystems) SNP browser were used to select tagging SNPs in the BRD1 locus. The screened region was extended 5 kb upstream of the annotated transcription start site and downstream at the end of the last *BRD1* exon [Neale and Sham, 2004]. The tagging SNP selection criteria were that polymorphisms had a minor allele frequency >5% in the Japanese population (release #21; phase II; July 2006). Then, we took advantage of observed linkage disequilibrium [Barrett et al., 2005] in the *BRD1* locus to exclude redundant SNPs from genotyping. In other words, if the correlation coefficient between two loci (r^2) was 0.9 or higher, then only one of the two loci was selected for the association study [Barrett et al., 2005]. Based on our criteria, 10 SNPs were selected for the analysis. The promoter SNP rs138880, which was one of the two SNPs associated with schizophrenia in the previous study [Severinsen et al., 2006], was included in these 10 SNPs. The 3′ UTR SNP rs4468, the other SNP associated with schizophrenia in the previous study, was also added to the tagging SNPs despite a lack of information on frequency of this polymorphism in a Japanese population in the HapMap database. Therefore, 11 SNPs made up the tagging set. All SNPs were genotyped by TaqMan assay (Applied Biosystems Japan Ltd, Tokyo, Japan). For quality control, three strategies were employed. First, we checked deviation from the Hardy–Weinberg equilibrium (HWE). Second, we genotyped 20 randomly selected samples for each SNP in duplicate in order to evaluate the genotype error rate. Third, we confirmed whether the minor allele frequency for each SNP genotyped in control samples was consistent with that in the Japanese population in the HapMap database.

Imputation of Ungenotyped SNPs

Because tagging SNPs was selected based on r^2, we included imputation as an exploratory method to compute genotypes of SNPs that were not selected for genotyping (untyped SNPs). The advantage of imputing untyped SNPs is that the coverage of common variants within the locus of interest can be enhanced, boosting the statistical power [Marchini et al., 2007]. The MACH program (http://www.sph.umich.edu/csg/abecasis/MACH/) was used to calculate the genotypic prediction of 20 untyped SNPs using directly typed SNP information (10 SNPs used in the screening scan) and the HapMap database (recombination map and haplotype data for the Japanese/Chinese population, release #23a; phase II; March 2008). The MACH program was recently reported to have similar imputation accuracy rates to IMPUTE and to outperform fastPHASE, PLINK, and Beagle [Pei et al., 2008]. The targeted region for imputation was limited to the *BRD1* locus as defined above.

Power Calculation

Power was calculated according to the methods described by Skol et al. [2006]. In brief, for a predefined alpha level, in the disease prevalence and inheritance model, statistical power of any given sample is a function of sample size and effect size. In other words, power is directly proportional to sample size on one side and minor allele frequency and genotype relative risk on the other side.

Statistical Methods for Association Study

Deviation from the HWE was tested by chi-square analysis. All single marker association analyses were done by calculating the

P-values for each SNP marker, and the significance was determined at the 5% level using the chi-square test, as implemented in SPSS v13 (SPSS, Inc., Chicago, IL). All *P*-values were two-sided. Multimarker analysis was carried out by log-likelihood ratio tests for assessing haplotype-wise associations between schizophrenia and a combination of tagging SNPs with a permutation test for calculating empirical significance levels for differences between haplotype frequencies in case and control subsets.

Meta-Analysis

We performed a meta-analysis for rs138880, one of the two SNPs associated with schizophrenia in the previous study [Severinsen et al., 2006]. The other SNP, rs4468, was excluded because it was not polymorphic in our sample. Thus far, only one study has been published regarding an association analysis of the *BRD1* locus [Severinsen et al., 2006]. We used data from Severinsen's study and our study. First, the Q statistic test was performed to assess the possible heterogeneity in the combined studies. Second, a fixed effects model meta-analysis was conducted. The significance of the overall odds ratio (OR) was determined by the Z-test. The analysis was carried out on Comprehensive Meta-Analysis software (Version 2.2.046, Biostat, Englewood, NJ).

Lymphoblastoid Cell Lines (LCLs)

Peripheral blood was drawn into 7-ml plastic tubes containing sodium heparin, and lymphocytes were separated by a standard protocol. The cells were cultured in RPMI-1460 medium containing 20% fetal bovine serum, penicillin, and streptomycin, and filtered supernatant of a B95-8 cell culture infected with Epstein–Barr virus. Cyclosporine A was added until colonies were observed. After colony formation, the cells were passaged three times per week, without the addition of 10% fetal bovine serum and cyclosporine A. The cells were frozen in liquid nitrogen until needed, at which time they were thawed, passaged at least three times, and used within 4 weeks. We paid special attention while establishing and maintaining cell lines to exclude environmental confounders as much as possible.

Real-Time Quantitative Polymerase Chain Reaction (PCR) and Statistical Analysis

Total RNA of LCLs was extracted using RNeasy Plus Mini kit (50) (Qiagen, Valencia, CA). RNA yield and quality were assessed by measuring absorbance at 260 and 280 nm. Integrity and overall quality of the total RNA preparation were determined by native agarose gel electrophoresis (inspection of the 28S and 18S bands). Total RNA was used for cDNA synthesis by High-Capacity cDNA Reverse Transcription Kits (Applied Biosystems Japan Ltd). Real-time quantitative PCR using TaqMan gene expression assays (Applied Biosystems Japan Ltd) was performed with ABI PRISM 7900HT (Applied Biosystems Japan Ltd). Amplification efficiency for each gene-specific primer pair was calculated based on the dilution series method [Livak and Schmittgen, 2001]. In each experiment, the r^2 value of the curve was more than 0.99. Measurement of the cycle threshold was performed in triplicate. The relative expression of *BRD1* was calculated by the modified ΔΔ cycle threshold method as implemented in Relative Expression Software Tool 2008 (REST 2008) [Pfaffl et al., 2002]. The normalization factor was the geometric mean [Vandesompele et al., 2002] of the following genes: tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein, zeta polypeptide (*YWHAZ*), beta-2-microglobulin (*B2M*), and ubiquitin C (*UBC*). These three genes were shown to have high expression stability in leukocytes [Vandesompele et al., 2002]. Bootstrapping techniques were used to provide 95% confidence intervals (CIs) for expression ratios without a normal or symmetrical distribution assumption.

RESULTS

Association Analysis

Regarding quality control, significant deviation from HWE was not observed. The genotypes of the duplicated samples showed complete concordance. Minor allele frequency for each tagging SNP in control samples generally showed a high concordance with that in HapMap database. Assuming a multiplicative model of inheritance and a disease prevalence of 1%, calculations showed that our sample had appropriate power (more than 80%) to detect gene-wide significant associations with genotype relative risk values from 1.24 to 1.55 (minor allele frequency values from 0.05 to 0.45). 3′ UTR SNP rs4468, which was associated with schizophrenia in a previous study, was not polymorphic in our Japanese sample, so we excluded rs4468 from subsequent analyses. Regarding the remaining 10 SNPs, no association was detected with schizophrenia in allele-/genotype-wise analyses or in the haplotype-wise analysis (two- to four-marker sliding window fashion; Table I). However, it should be noted that the rs138880 (associated SNP in previous article) showed the same trend in the Japanese population. In addition, haplotype showing the most significant association [Severinsen et al., 2006] was tested in the present study. We could not show a significant difference in the frequency of this haplotype between cases and controls (haplotype frequency in cases and controls: 0.0010 and 0.0010, respectively, $P = 0.99$).

Imputation of Ungenotyped SNPs

We used MACH to infer genotypes of 20 untyped SNPs. We provided genotypes for our own data (10 SNPs) as input together with haplotypes from the HapMap Japanese/Chinese population. The imputation method using MACH did not support an association between schizophrenia and the 20 SNPs in the *BRD1* locus (Table II).

Meta-Analysis

The SNP rs138880 that previously has been associated with schizophrenia showed the same trend in the Japanese population although it did not reach significance. The ORs for rs138880 reported in the Severinsen et al. [2006] and in this study were 1.73 and 1.14, respectively (Supplementary Table III). The pooled OR derived from the two studies (in total, 729 cases and 970 controls) was significant in the fixed model (pooled OR = 1.25, 95% CI = 1.03–1.52, $P = 0.02$; Supplementary Table III). Homogeneity analysis for the OR

TABLE I. Allele-Wise, Genotype-Wise, and Haplotype-Wise Analyses of 10 Tagging Single-Nucleotide Polymorphisms (SNPs)

	Allele frequency (proportion)				Single SNP		Haplotype wise		
	Case		Control						
dbSNP	M	m	M	m	Allele-wise	Genotype-wise	2-window[a]	3-window[a]	4-window[a]
rs138820	0.78	0.22	0.78	0.22	0.86	0.09			
							0.83		
rs4469	0.77	0.23	0.78	0.22	0.45	0.75		0.7	
							0.29		0.7
rs6009874	0.92	0.08	0.92	0.08	0.92	0.15		0.62	
							1.00		0.69
rs138840	0.92	0.08	0.92	0.08	0.82	0.92		1.00	
							1.00		0.47
rs138844	0.84	0.16	0.84	0.16	0.69	0.37		0.49	
							0.54		0.36
rs138850	0.59	0.41	0.59	0.41	0.82	0.25		0.46	
							0.52		0.44
rs138851	0.94	0.06	0.95	0.05	0.14	0.31		0.48	
							1.00		0.81
rs138863	0.94	0.06	0.95	0.05	0.15	0.38		0.71	
							0.68		1.00
rs2239848	0.85	0.15	0.86	0.14	0.79	0.72		1.00	
							1.00		
rs138880	0.86	0.14	0.87	0.13	0.22	0.09			

M, major allele; m, minor allele.
[a]Sliding window analysis, rare haplotype threshold 10%.

TABLE II. Allele-Wise Analysis of 20 Imputed Single-Nucleotide Polymorphisms

dbSNP	*P*-value	Quality[a]
rs138816	0.97	0.92
rs138821	0.91	0.96
rs2269626	0.72	0.95
rs138823	0.91	0.95
rs916418	0.96	0.99
rs916419	0.85	0.99
rs138827	0.82	0.99
rs138830	0.81	0.99
rs138834	0.87	0.99
rs138841	0.37	0.99
rs138843	0.86	1.00
rs138845	0.34	0.99
rs6009878	1.00	0.99
rs138853	0.14	1.00
rs138861	0.41	1.00
rs138866	0.27	0.99
rs138867	0.27	1.00
rs138870	0.27	0.99
rs138871	0.23	0.99
rs138884	0.23	1.00

[a]Quality is the average posterior probability for the most likely genotype.

revealed no significant evidence for heterogeneity of the OR ($Q = 2.98$, $df = 1$, $P = 0.084$).

Expression Analysis

The expression of *BRD1* mRNA was analyzed using LCLs from 29 cases and 30 controls. Cycle threshold values of *BRD1* and three internal controls (*B2M*, *UBC*, and *YWHAZ*) are shown in Supplementary Table IV. We could not detect any significant differences in *BRD1* mRNA levels between cases and controls ($P = 0.46$; Fig. 1).

DISCUSSION

The common disease–common variant hypothesis states that diseases that were evolutionarily neutral (i.e., had little or no effect on reproductive fitness), such as late-onset schizophrenia, during human history may be significantly influenced by common variants [Lander, 1996]. Therefore, if allelic variants at a disease susceptibility locus are responsible for the predisposition to a common complex disease, then allele-, genotype-, or haplotype-wise association tests will detect such variants (or tagging SNPs that are in linkage disequilibrium with the deleterious allele).

The first and only indication that the *BRD1*-related region harbors a variation that might influence susceptibility to schizophrenia was provided by Severinsen et al. [2006], who identified two fairly strong association signals between two SNPs (rs4468 and rs138880) and schizophrenia using a case–control sample from Scotland. The sample in this study consisted of 103 patients with

(i)

Gene	Type	95% C.I.	P(H1)
BRD1	Target	0.56 - 2.08	0.46

(ii)

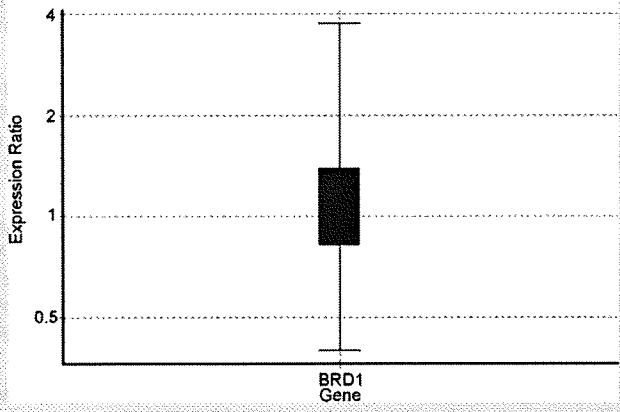

FIG. 1. Relative expression of bromodomain-containing 1 (BRD1) normalized to the geometric mean of three internal controls. I: The relative expression of BRD1 was normalized to the geometric mean of three internal controls (B2M, UBC, and YWHAZ). Bootstrapping techniques were used to provide 95% confidence intervals for expression ratios without normal or symmetric distribution assumption. The number of iterations was 10,000 in this analysis. *P* (H1) means the probability of the alternate hypothesis that the difference between sample and control groups is due only to chance. II: Boxplot. Expression ratio is the relative expression of BRD1 in cases compared with controls (expression in control is equivalent to 1). Box represents the middle 50% of observations. The dotted line represents the median gene expression. Whiskers represent the minimum and maximum observations.

schizophrenia and 200 controls. Our study did not strongly support an association between schizophrenia and the *BRD1* locus although the only previously associated SNP included in our study (rs138880) showed the same trend, and the meta-analysis of this SNP using a fixed effects model was significant.

Psychiatric disorders are complex diseases that are characterized by the contribution of multiple susceptibility genes and environmental factors. Therefore, *BRD1* might be a population-specific factor for schizophrenia. However, this conclusion should be made only with the following considerations. First, it is possible that our study was still underpowered to reliably detect common low-risk variants. This may be related to etiological heterogeneity or inaccurate diagnoses in schizophrenia, which would attenuate the genetic relative risk. Second, only the hypothesis of an association with common SNPs of *BRD1* has been tested, both here and in the previous study; therefore, future studies using resequencing methods to detect rare variants in the *BRD1* locus will be needed for a complete understanding of relationship between this genetic locus and schizophrenia. Third, even though the Japanese population is relatively homogeneous [Haga et al., 2002], small population stratifications may have affected our findings. A recent analysis with the use of approximately 140,000 SNPs in 7003 Japanese individuals has shown that local regions within the main island of Japan are genetically differentiated in spite of frequent human migration within Japan in modern times [Yamaguchi-Kabata et al., 2008]. However, we believe that the impact of population stratification on our study is negligible, as our samples were collected in a relatively narrow region in the middle of the main island of Japan. Fourth, regarding the Japanese and the Caucasian populations, comparative linkage disequilibrium analysis of the HapMap data showed a different block structure around the *BRD1* locus [Gabriel et al., 2002]. Compared with the Caucasian population, linkage disequilibrium (LD) blocks in the Japanese population are shorter, and the block structure is coarser, having lower r^2 values. This might influence interpopulation transferability of tagging SNPs in the *BRD1* locus and result in a failure to detect an association with schizophrenia in the Japanese population. Interestingly, selective sweep analysis has provided evidence of recent positive selection on genes associated with schizophrenia, and *BRD1* gene was reported to have been affected by positive selection in Caucasian but not in Asian population [Crespi et al., 2007]. This indicates that the positive selection specific to the Caucasian population might produce the difference in LD structure in *BRD1* locus.

We could not detect significant differences in *BRD1* mRNA levels between cases and controls in the expression analysis. These results are consistent with the findings in the association study. However, there were several limitations in the expression assays. Using non-neuronal samples such as LCLs is based on the assumption that heritable mechanisms associated with the risk of schizophrenia have systemic effects and result in changes to gene expression in various tissues. To validate the use of gene expression data in a more accessible tissue as a surrogate for gene expression in the central nervous system, Sullivan et al. [2006] evaluated the comparability of transcriptional profiling of a variety of human tissues with Affymetrix U133A microarray augmented with a custom microarray. Their analyses suggested that careful use of peripheral gene expression may be a useful surrogate for gene expression in the central nervous system.

In conclusion, we could not strongly show that common SNPs in the *BRD1* gene account for a substantial proportion of the genetic risk for schizophrenia in the Japanese population, although small effects of population stratification or differences in LD structure could not be ruled out. Considering the significance in the meta-analysis for the only previously associated SNP included in our study, further investigations are needed for conclusive results.

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