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Analysis of methylation and -141C *Ins/Del* polymorphisms of the dopamine receptor D2 gene in patients with schizophrenia

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ABSTRACT

The gene for dopamine receptor D2 (DRD2) is associated with schizophrenia (SCZ). Epigenetic changes may be related to SCZ pathology. The -141C *Ins/Del* polymorphism in DRD2 (rs1799732) is functional and associated with SCZ. Fifty SCZ patients and 50 control subjects were newly recruited and analyzed in addition to 50 previously reported SCZ samples and 50 previously reported control samples. Genomic DNA from peripheral leukocytes was analyzed. We replicated analysis of DNA methylation rates at seven CpG sites (CpG 1-1 to 1-7) and also analyzed five additional sites (CpG 2-1 to 2-5) in the upstream region of DRD2. We also performed genotyping of -141C I*ns/Del* and analyzed the effects of -141C *Ins/Del* on methylation of DRD2. Methylation rates were significantly lower in SCZ patients compared to control subjects, respectively. In control subjects, the methylation rates were significantly lower in individuals with the *Ins/Ins* genotype than in Del allele carriers. We replicated hypomethylation of the DRD2 promoter region in SCZ patients compared to age-matched control subjects. The -141C *Ins/Del* polymorphism affected the methylation rates in regions of DRD2. Hypomethylation and the -141C *Ins/Del* polymorphism of DRD2 may be biomarkers for SCZ.

1. Introduction

Patients with schizophrenia (SCZ), a common mental disorder, show various symptoms such as hallucinations, delusions, disorganized communication, poor planning, reduced motivation, and blunted affect (van Os, 2009; Kahn et al., 2015). SCZ has a complex etiology, involving both genetic and environmental factors (Sullivan et al., 2003). Based on the dopamine hypothesis, various antipsychotics that antagonize the dopamine receptor D2 (*DRD2*) have been developed for treatment of SCZ (Seeman, 1975; Creece, 1976; Lee, 1980; Strange, 2001). A significant association between *DRD2* and development of SCZ was reported by a large-scale genome-wide association study (Schizophrenia Working Group of the Psychiatric Genomics Consortium, 2014).

Several imaging studies have shown an increase in D2 receptor density in the striatum in SCZ patients compared to that in control subjects (Brunelin et al., 2013; Kubota et al., 2017). Epigenetic changes including DNA methylation, which is associated with gene expression, regulate neurodevelopment and psychopathological changes in SCZ (Abdolmaleky et al., 2004; Cholewa-Waclaw et al., 2016; Goud Alladi, 2018). However, a definitive consensus on DNA methylation of *DRD2* by using peripheral leukocytes has not been reached. Using peripheral leukocytes from SCZ patients compared to control subjects, Zhang et al. (2007) reported no significant changes in SCZ patients, whereas Yoshino et al. (2016) showed hypomethylation of *DRD2* in SCZ patients. Hypomethylation of *DRD2* may affect gene expression and may be related to SCZ pathology (Brunelin et al., 2013; Kubota et al., 2017).

Meta-analysis of relationships between the -141C *Ins/Del* (rs1799732) polymorphism in *DRD2* and the risk of SCZ showed that Asian *Del* allele carriers are not likely to develop SCZ (He et al., 2016). However, *DRD2* polymorphisms such as -141C *Ins/Del* and Taq1A are associated with treatment response in SCZ (Zhang et al., 2010). Several studies indicate that *Del* allele carriers are likely to show a poor response and to develop extrapyramidal symptoms or tardive dyskinesia with antipsychotic treatment (Inada et al., 1999; Zhang et al., 2010). Using a cell line, Arinami et al. (1997) reported that -141C *Ins/Del*, which is located in the 5′-promoter region of *DRD2*, may be a functional polymorphism. In control subjects, DRD2 density in the striatum is increased in *Del* allele carriers as shown with a positron emission

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Table 1
Schizophrenia patients and control subjects.

	Ct subjects (n = 100)	SCZ patients (n = 100)	p value
Sex (male:female)	49:51	48:52	1
Age (years)	58.6 ± 15.4	58.7 ± 14.9	0.947
-141C *Ins/Del*			
Ins/Ins	69	74	
Ins/Del	29	25	
Del/Del	2	1	

Values denote the mean ± standard deviation.
Abbreviations: Ct, control; SCZ, schizophrenia,.
-141C *Ins/Del*; -141C *Ins/Del* polymorphisms of *DRD2*.

tomography (PET) study *(in vivo)* (Jonsson et al., 1999). However, Smith et al. (2017) did not find a statistically significant difference in different -141C *Ins/Del* genotypes when performing a similar study.

Although several studies indicated significant associations between single nucleotide polymorphisms and DNA methylation (Docherty et al., 2012; Rajala et al., 2014), the effects of the *DRD2*-141C *Ins/Del* polymorphism on methylation rates has not been studied. Thus, the purposes of this study were as follows. First, we replicated the study by Yoshino et al. (2016), who examined seven CpG sites in *DRD2*, by adding 100 new samples (50 SCZ patients and 50 control subjects) to the analysis. Second, we analyzed an additional five CpG sites adjacent to the -141C *Ins/Del* polymorphism using 200 samples. Third, we analyzed the effects of the -141C *Ins/Del* polymorphism on methylation rates (12 CpG sites).

2. Methods

2.1. SCZ patients and control subjects

Demographic data for each group of participants are shown in Table 1. We added 50 newly recruited SCZ patients to the samples previously reported by Yoshino et al. (2016). Thus, a total of 100 SCZ patients were recruited from Ehime University Hospitals in Japan. SCZ patients were diagnosed by at least two expert psychiatrists based on DSM-5 criteria. Clinical parameters of SCZ patients are shown in Table 2. Regarding the additional 50 SCZ patients, we evaluated SCZ symptoms using the Positive and Negative Syndrome Scale (PANSS) (Kay et al., 1987). **Analysis of 18-item Brief Psychiatric Rating Scale (BPRS) (each item is scored on a 1–7 scale) was conducted using 49 patients that had been previously reported (**Yoshino et al., 2016; Rhoades, 1988**). In addition, we also evaluated SCZ symptoms for these 50 patients with the BPRS in PANSS (**Rhoades, 1988**). Total 99 patients were evaluated by BPRS.** We evaluated antipsychotic-induced extrapyramidal symptoms using the Drug-Induced Extrapyramidal Symptoms Scale (DIEPSS) (Inada, 2009). Analysis of each clinical parameter was conducted using 100 samples including 50 samples that had been previously reported (Yoshino et al., 2016). We also added 50 newly recruited control subjects, for a total of 100 age-matched control subjects who were free from psychiatric problems, past histories of mental illness, and medications (Table 1). All participants were unrelated, of Japanese origin, and provided written informed consent forms approved by the institutional ethics committees of Ehime University Hospital.

2.2. Collection of blood samples

We collected venous blood using potassium EDTA tubes. Genomic DNA (gDNA) samples were collected from whole peripheral blood leukocytes. The gDNA was extracted using a blood mini kit with QIAcube (Qiagen, Tokyo, Japan) according to the manufacturer's protocol. We stored purified gDNA at 4 °C until genetic analyses.

Table 2
Demographic data of schizophrenia patients.

Characteristics		CpG 1-1	CpG 1-2	CpG 1-3	CpG 1-4	CpG 1-5	CpG 1-6	CpG 1-7	Average of CpG 1-1 to 1-7	CpG 2-1	CpG 2-2	CpG 2–3	CpG 2–4	CpG 2–5	Average of CpG 2–1 to 2–5
Age (years, n = 100)	58.7 ± 14.9	0.203	0.122	***0.328**	0.225	0.225	***0.286**	0.077	***0.296**	0.170	0.112	−0.038	0.018	0.078	0.109
Age of onset (years, n = 98)	28.1 ± 11.8	−0.081	0.082	0.235	0.183	0.111	0.121	−0.070	0.154	0.167	0.104	0.040	0.070	0.195	0.162
Duration (years, n = 98)	31.0 ± 14.3	0.258	0.125	0.193	0.080	0.182	0.198	0.204	0.223	0.020	0.056	−0.052	−0.043	−0.087	−0.005
CP equation (n = 99)	680.9 ± 579.8	0.018	−0.086	−0.088	−0.031	0.024	0.046	0.058	−0.051	−0.137	−0.113	−0.017	−0.070	−0.097	−0.111
BPRS (n = 99)	37.7 ± 12.8	−0.144	0.224	−0.023	0.136	0.248	0.095	***0.340**	0.150	−0.069	0.104	0.213	0.074	0.085	0.040
PANSS (n = 50)															
Total	77.7 ± 18.3	0.257	−0.066	−0.214	−0.153	−0.083	−0.094	−0.150	−0.129	−0.143	−0.081	−0.079	−0.115	−0.158	−0.144
Positive	18.1 ± 5.6	0.123	−0.074	−0.269	−0.150	−0.074	−0.010	−0.178	−0.160	−0.187	−0.049	−0.009	−0.218	−0.041	−0.164
Negative	20.2 ± 5.2	0.233	−0.230	−0.275	−0.205	−0.167	−0.179	−0.210	−0.229	−0.153	−0.052	0.001	0.018	−0.057	−0.081
General	39.3 ± 9.8	0.192	0.015	−0.106	−0.087	−0.014	−0.087	−0.130	−0.055	−0.099	−0.104	−0.172	−0.117	−0.250	−0.154
DIEPSS (n = 98)	4.6 ± 3.6	0.122	−0.008	0.097	0.069	0.147	0.102	0.082	0.100	0.138	0.138	0.035	0.048	0.125	0.125

Values denote the mean ± standard deviation and correlation coefficient (r).
We could not analyze some samples due to a lack of clinical data. For the 50 newly added samples, we used BPRS in PANSS. Correlation of the methylation rate of each CpG site with some parameters was conducted with Spearman's rank correlation coefficient. All correlations were statistically significant even after Bonferroni correction (p = 0.004).
Abbreviations: Duration, duration of illness; CP equivalent, chlorpromazine equivalent; BPRS, Brief Psychiatric Rating Scale; PANSS, Positive and Negative Syndrome Scale; DIEPSS, Drug Induced Extra-Pyramidal Symptoms Scale.

2.3. Bisulfite conversion

Bisulfite conversion of gDNA (1000 ng each) was performed using the EpiTect Plus DNA Bisulfite Kit (Qiagen, Valencia, CA, USA). Converted gDNA was purified according to the protocols in this kit.

2.4. PCR amplification

We used the same primer pair for CpG 1-1 to 1-7 as reported previously (Yoshino et al., 2016). The final volume of each PCR sample was 40 µl, and each reaction contained 3.5 µl bisulfite-converted gDNA, 0.2 mM dNTPs, 10 × PCR buffer, 0.5 U AmpliTaq gold (Applied Biosystems, Foster City, CA, USA), and 0.2 µM forward and reverse primers. In addition, we analyzed five new CpG sites (CpG 2-1 to 2-5) using the following primers: forward 5′-AGTAGAAGGTGAGGATTTAG TTTGTAAT-3′, reverse 5′-CCCCACCAAAAAAACTATACCTCC-3′. The final volume of each PCR sample was 25 µl, and each reaction contained 2.0 µl bisulfite-converted gDNA. The final concentration of the other reagents was the same as above. The thermal cycling conditions included an initial denaturation for 10 min and 45 subsequent cycles of denaturation for 30 s at 95 °C, annealing for 30 s at 60 °C, elongation for 1 min at 72 °C, and a final extension at 72 °C for 10 min. We combined two sets of PCR products by using the Fast Gene Gel/PCR Extraction Kit (NIPPON Genetics, Japan) after PCR amplification when sufficient peaks were not obtained.

2.5. Measurement of methylation rates

We analyzed the seven previously reported CpG sites and the additional five CpG sites (Fig. 1) (Yoshino et al., 2016). The PCR products were sequenced with PyroMark Q24 Advanced (Qiagen, Tokyo, Japan). The sequencing primers were: 5′-AGTTTTTAAAGGAGAAGATT-3′ (CpG 1-1 to 1-7) and 5′-GTTTTAGTTTTAAAATAAGGGATGG-3′ (CpG 2-1 to 2-5). We analyzed each CpG methylation rate in duplicate. Then, each methylation rate was quantified using PyroMark Q24 Advanced software.

2.6. Genotyping

We performed genotyping of polymorphisms (-141C *Ins/Del*; rs1799732) in *DRD2* using the TaqMan 5′-exonuclease allelic discrimination assay (Assay ID: C_33641686_10, Applied Biosystems) using the StepOnePlus real-time PCR system (Applied Biosystems, Tokyo, Japan). All genotypes of -141C *Ins/Del* were determined automatically using the StepOnePlus real-time PCR system. The genotyping call rate was 100%.

2.7. Statistical analysis

We conducted statistical analyses using SPSS 22.0 software (IBM Japan, Tokyo, Japan). We used a test of normality with the Shapiro–Wilk test. Because methylation rates and clinical parameters did not follow the normal distribution, we should use a non-parametric test. Comparisons of each CpG methylation rate in *DRD2* between SCZ patients and control subjects were performed with the Mann–Whitney *U* test. We assessed sex and -141C *Ins/Del* genotype differences using Fisher's exact test. Comparisons of age between SCZ patients and control subjects were analyzed with the Mann–Whitney *U* test. Correlations between each clinical parameter and the methylation rate were conducted with Spearman's rank correlation coefficient. Comparisons between -141C *Ins/Del* and DIEPSS were analyzed using multiple logistic regression analysis after adjusting for sex, age, age of onset, duration, chlorpromazine (CP) equivalent, and BPRS. Statistical significance was defined at the 95% level ($p = 0.05$). We used Bonferroni corrections as post-hoc tests when examining the comparisons of each CpG methylation rate between SCZ patients and control subjects.

3. Results

3.1. Participant characteristics

Demographic data for each group of participants are shown in Table 1. SCZ patients and control subjects did not differ in sex ($p = 1$) or age ($p = 0.947$). We found no significant difference in the -141C *Ins/del* genotype (-141C *Ins/Ins* or *Del* allele carrier, Fisher's exact test; $p = 0.531$) between SCZ patients and control subjects.

3.2. Comparisons of methylation rates between SCZ patients and control subjects

The results of the methylation comparison are shown in Fig. 2a and b. For the seven CpG sites previously studied (Yoshino et al., 2016), CpG 1-2 (12.8 ± 3.0 vs. 14.0 ± 2.6, $p = 0.001$), 1–4 (25.8 ± 4.0 vs. 27.8 ± 4.0, $p = 0.003$), 1-5 (7.8 ± 1.7 vs. 8.5 ± 1.8, $p = 0.005$), 1–6

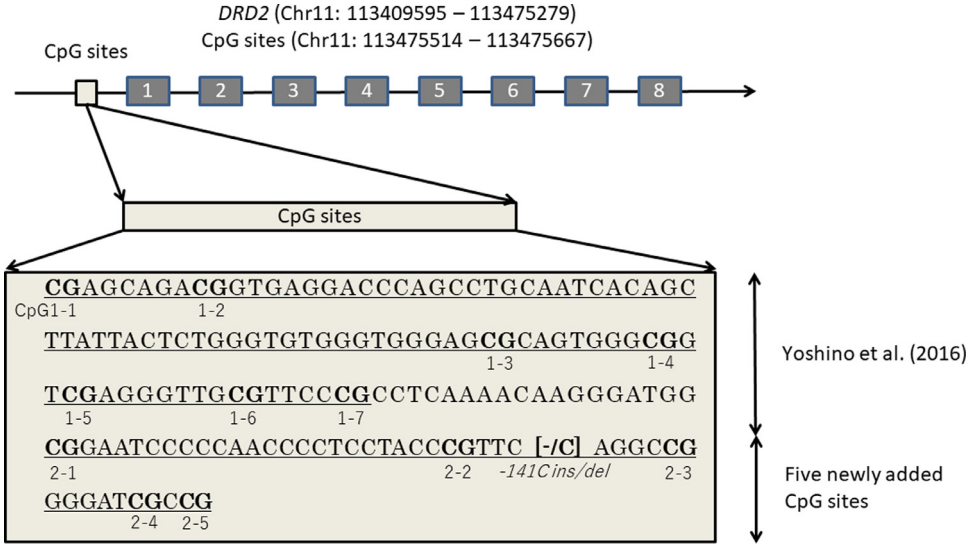

Fig. 1. The diagram shows the location of the *DRD2* promoter regions we analyzed. Seven CpG sites (CpG 1-1 to 1-7) were previously analyzed by Yoshino et al. (2016), and we added five new CpG sites (CpG 2-1 to 2-5). Abbreviations: *DRD2*, dopamine receptor, D2 gene.

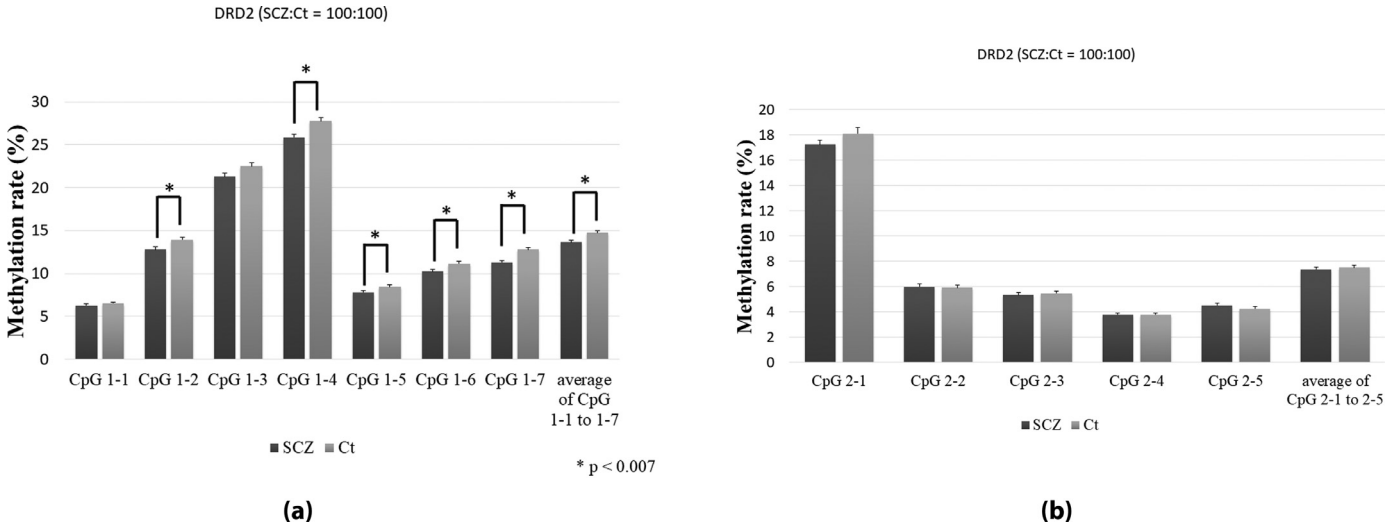

Fig. 2. a and b. Comparisons of methylation rates of *DRD2* between SCZ patients and control subjects. Each bar shows the mean ± standard error. Statistical significance was defined at $p = 0.007$ (a) and $p = 0.01$ (b) after Bonferroni correction (Mann–Whitney *U* test). Abbreviations: SCZ, schizophrenia patients; Ct, control subjects.

(10.3 ± 1.9 vs. 11.1 ± 2.3, $p = 0.005$), 1-7 (11.3 ± 2.2 vs. 12.8 ± 2.4, $p < 0.001$), and the average of CpG 1-1 to 1-7 (13.7 ± 1.9 vs. 14.7 ± 2.2, $p = 0.001$) were significantly lower in SCZ patients than in control subjects, respectively, after Bonferroni correction ($p < 0.007$). The methylation rates tended to be decreased in SCZ patients at all CpG sites of CpG 1-1 to 1-7. Additionally, we analyzed five new CpG sites. However, we found no significant differences in methylation for CpG 2-1 to 2-5 between control and SCZ subjects. We also found no significant differences in the average of CpG 2-1 to 2-5 (7.4 ± 1.7 vs. 7.5 ± 2.0, $p = 0.272$). When we analyzed only the new 100 samples (50 SCZ patients and 50 control subjects), we reconfirmed the significantly lower methylation rates in SCZ patients compared to control subjects at CpG 1-6 (10.8 ± 1.6 vs. 12.1 ± 2.0, $p < 0.001$), 2-1 (16.7 ± 3.1 vs. 18.6 ± 4.5, $p = 0.009$), and the average of CpG 1-1 to 1-7 (14.4 ± 1.7 vs. 15.4 ± 1.9, $p = 0.012$).

3.3. Comparisons of methylation rates between Ins/Ins and Del allele carriers in control subjects

We compared methylation rates of *DRD2* in control subjects between -141C *Ins/Ins* and *Del* allele carriers (Fig. 3a and b). *Ins/Ins* ($n = 69$; 33 males, 36 females; age = 59.2 ± 15.5 years) and *Del* allele carriers ($n = 31$; 16 males, 15 females; age = 57.4 ± 15.4 years) among control subjects did not differ according to sex ($p = 0.830$) or age ($p = 0.563$). The methylation rates at CpG 1-3 (21.6 ± 4.3 vs. 24.6 ± 3.7, $p < 0.001$) and the average of CpG 1-1 to 1-7 (14.4 ± 2.2 vs. 15.4 ± 1.9, $p = 0.024$) were significantly lower in *Ins/Ins* than *Del* allele carriers, respectively, in control subjects. The methylation rates at CpG 2-1 (17.4 ± 5.1 vs. 19.6 ± 3.5, $p = 0.006$) were also significantly lower in *Ins/Ins* than *Del* allele carriers, respectively, in control subjects.

3.4. Comparisons of methylation rates between Ins/Ins and Del allele carriers in SCZ patients

We compared methylation rates of *DRD2* in leukocytes from SCZ patients between -141C *Ins/Ins* and *Del* allele carriers (Fig. 4a and b). *Ins/Ins* ($n = 74$; 36 males, 38 females; age = 58.7 ± 14.9 years) and *Del* allele carriers ($n = 26$; 12 males, 14 females; age = 58.7 ± 15.2 years) among SCZ patients did not differ according to sex ($p = 0.807$) or age ($p = 1$). The methylation rates at CpG 2-1 (16.8 ± 3.4 vs. 18.6 ± 3.5, $p = 0.008$) were significantly lower in *Ins/Ins* compared to *Del* allele carriers, respectively, in SCZ patients. We found no significant differences in any CpG sites from CpG 1-1 to 1-7. We also found no

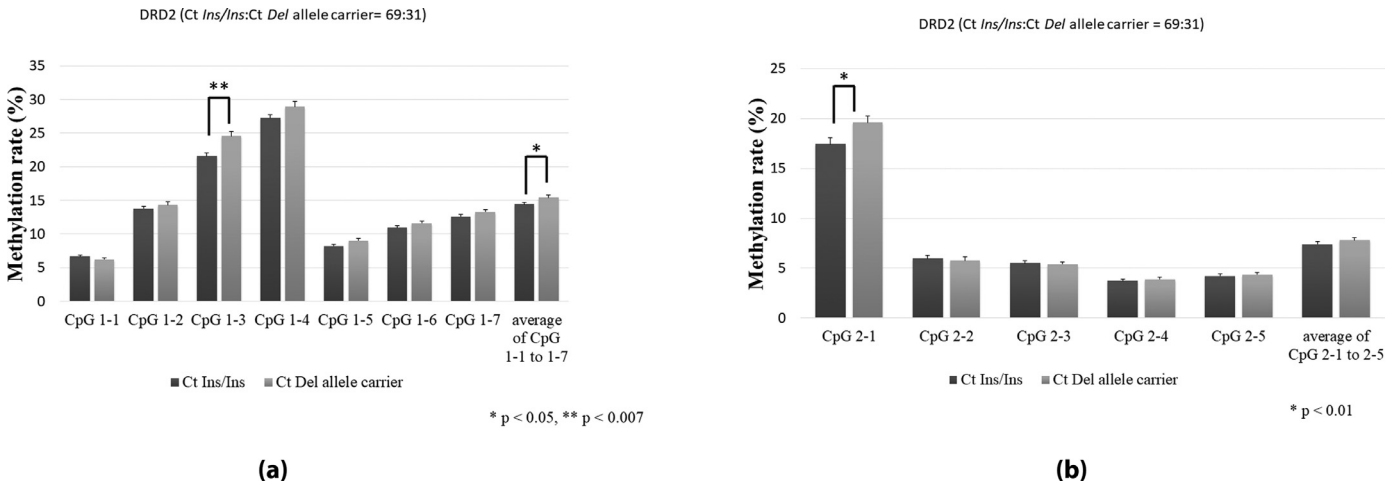

Fig. 3. a and b. Comparisons of methylation rates of *DRD2* between Ct *Ins/Ins* and Ct *Del* allele carriers. Each bar shows the mean ± standard error. Statistical significance was defined at $p = 0.007$ (a) and $p = 0.01$ (b) after Bonferroni correction (Mann–Whitney *U* test). Abbreviations: Ct, control subjects.

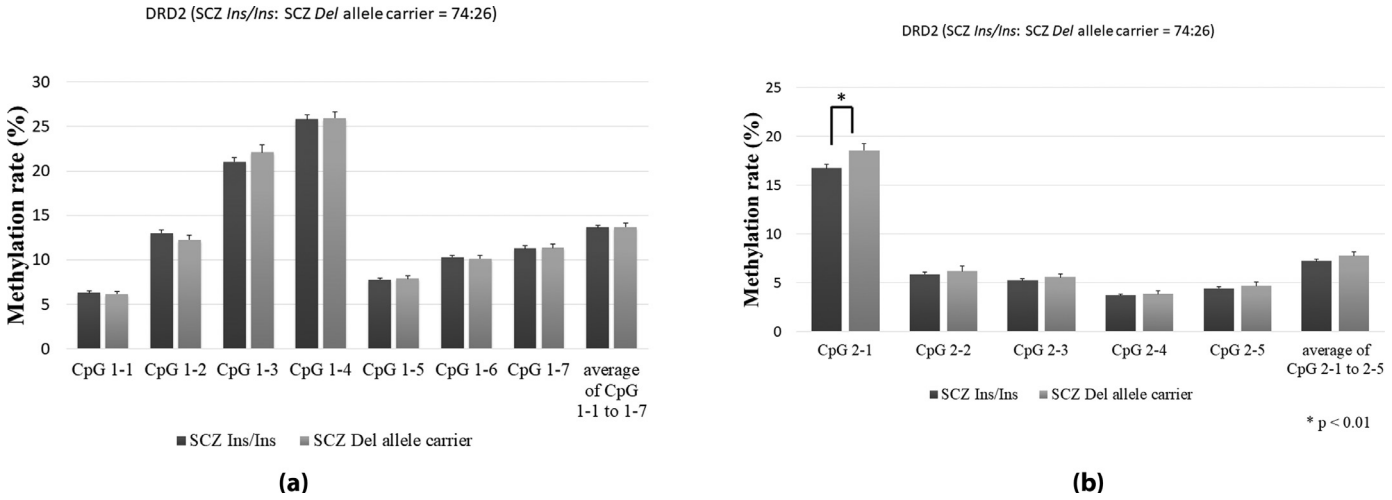

Fig. 4. a and b. Comparisons of methylation rates of *DRD2* between SCZ *Ins/Ins* and SCZ *Del* allele carriers. Each bar shows the mean ± standard error. Statistical significance was defined at $p = 0.007$ (a) and $p = 0.01$ (b) after Bonferroni correction (Mann–Whitney *U* test). Abbreviations: SCZ, schizophrenia patients.

differences in the average of CpG 1-1 to 1-7 (13.7 ± 1.9 vs. 13.7 ± 2.1, $p = 0.735$).

3.5. Correlation between methylation rates and clinical parameters in SCZ patients

Almost all methylation rates were correlated with each other in both SCZ patients and control subjects (Suppl. fig. 1). Demographic data for clinical parameters in SCZ patients is shown in Table 2. We found a significant positive correlation between age and methylation rates at CpG 1-3 ($r = 0.328$, $p = 0.001$), CpG 1-6 ($r = 0.286$, $p = 0.004$), and the average of CpG 1-1 to 1-7 ($r = 0.296$, $p = 0.003$) after Bonferroni correction ($p = 0.004$). We also found a significant positive correlation between BPRS and methylation rates at CpG 1-7 ($r = 0.340$, $p = 0.001$).

3.6. Comparisons between -141C Ins/Del and DIEPSS in SCZ patients

We compared -141C *Ins/Del* and DIEPSS in SCZ patients using multiple logistic regression analysis. We adjusted for sex, age, age of onset, duration, CP equation, and BPRS. We excluded four patients due to a lack of clinical data, and thus, 96 SCZ patients (-141C *Ins/Ins:Del* allele carriers; 71:25) were included in this analysis. *Del* allele carriers were significantly associated with an increased DIEPSS score (odds ratio: 1.151 [95% CI: 1.013–1.308], **adjusted *p* value = 0.031 for multiple logistic regression analysis, original *p* value = 0.028 for Mann–Whitney *U* test**).

4. Discussion

Our study revealed two notable findings. First, the methylation rates of *DRD2* were significantly lower in SCZ patients and were correlated with BPRS. Second, -141C *Ins/Del* polymorphisms were associated with these methylation rates and DIEPSS.

DRD2 mRNA expression in leukocytes was too low to measure accurately with real-time PCR. Therefore, examination of methylation rates of the CpG sites (especially in CpG islands) in *DRD2* in leukocytes as a biomarker is useful because methylation generally plays an important role in gene expression (Fryxell, 2005). Epigenetic changes including DNA methylation reflect psychopathology, and thus, epigenetic changes may be related to SCZ pathogenesis and may be a biomarker in SCZ (Abdolmaleky et al., 2004). We analyzed seven CpG sites previously reported by Yoshino et al. (2016). Our replication study using leukocytes of seven CpG sites showed that the methylation rates of CpG 1-2, 1-4, 1-5, 1-6, 1-7, and the average of CpG 1-1 to 1-7 were lower in SCZ patients, confirming the results in a previous study (Yoshino et al., 2016). We examined an additional five CpG sites of *DRD2*. The methylation rates at these sites also tended to be decreased in SCZ patients at CpG 2-1. However, we found no significant differences in any CpG sites from CpG 2-1 to 2-5. These results indicate that a significant change in *DRD2* methylation in SCZ may not be observed along the entire promoter region. The methylation rates of genes in several tissues such as leukocytes are affected by age (Bollati et al., 2009; Christensen et al., 2009; Horvath et al., 2012). We found a significant positive correlation between age and methylation rates at CpG 1-3, CpG 1-6, and the average of CpG 1-1 to 1-7, which was consistent with a previous study (Yoshino et al., 2016). *DRD2* expression decreases in the brain with age. Therefore, hypermethylation of *DRD2* with age may reflect the decrease in *DRD2* expression (Karrer et al., 2017).

We found a significant positive correlation between BPRS and methylation rates at CpG 1-7. The mRNA expression levels of the serotonin transporter gene are associated with methylation of its CpG islands (Iga et al., 2016), and hypermethylation of the serotonin transporter gene may be related to psychiatric conditions (Philibert et al., 2007; Won et al., 2016). The higher methylation of *DRD2* in our study may also reflect a worse psychiatric condition. Although we found a significant positive correlation between BPRS and PANSS scores, we did not find a significant correlation between PANSS and methylation rates, which may due to the small number of patients assessed with PANSS.

Arinami et al. (1997) showed *in vitro* that the transcriptional strength at promoter sites of *DRD2* is decreased in a fragment that contains the -141C *Del* allele compared to a fragment that contains the *Ins* allele, and these authors indicated that this polymorphism is associated with SCZ. On the other hand, Jönsson et al. (1999) reported that regulation of *DRD2* density in the striatum is increased in *Del* allele carriers *in vivo* in a PET study. However, this PET study was conducted with a small sample size, and no difference was found in another study (Smith et al., 2017). Although several reports have shown that *Del* allele carriers are less frequent in SCZ patients than in control subjects (Cordeiro et al., 2009; Xiao et al., 2013; He et al., 2016), we did not find significant differences in -141C *Ins/Del* (rs1799732) carriers in this study, probably because of the relatively small sample size.

The methylation rates at CpG 1-3, 2-1, and the average of CpG 1-1 to 1-7 were significantly lower in *Ins/Ins* compared to *Del* allele carriers among control subjects, but we found no difference in CpG 2-2 to 2-5. The methylation rates at CpG 2-1 were also significantly lower in *Ins/Ins* than in *Del* allele carriers among SCZ patients. -141C *Ins/Del* seemed to affect methylation rates in regions upstream rather than downstream of -141C *Ins/Del*. *Del* allele carriers of -141C *Ins/Del* were associated

with an increased DIEPSS score. Several studies have indicated that the risk of extrapyramidal symptoms and tardive dyskinesia appears to be higher in SCZ patients who are *Del* allele carriers (Inada et al., 1999). *DRD2* expression levels were lower in *Del* allele carriers, and therefore, DRD2 activity may be blocked earlier by antipsychotic drugs. As a result, extrapyramidal symptoms may be more likely to occur in *Del* allele carriers.

Our study has several limitations. Whether the methylation rates of *DRD2* in leukocytes are correlated with those in the brain is unknown, although the methylation rate was reported to be correlated between those tissues (Davies et al., 2012; Wockner et al., 2015). Examination of methylation rates as biomarkers may be useful in SCZ, and comparison of the methylation rates and mRNA expression levels between SCZ patients and control subjects using brain samples will provide clarity about the usefulness of examining methylation rates in leukocytes. Second, we did not examine other polymorphisms such as the TaqA1 polymorphism, which may contribute to regulation of the methylation rates in *DRD2*. Third, we could not analyze all CpG sites in *DRD2* regarding whether -141C *Ins/Del* contributed to regulation of the methylation rates in *DRD2*. Lastly, we did not assess environmental effects such as medication on *DRD2* methylation rates. Yoshino et al. (2016) showed that hypomethylation of *DRD2* in SCZ patients was not different in the presence or absence of antipsychotic treatment. However, several studies have indicated that DNA methylation of *DRD2* changes in the presence of antipsychotic drugs (Melka et al., 2014; Murata et al., 2014). Further studies are needed to answer these questions.

In conclusion, we confirmed the hypomethylation rates of the *DRD2* promoter region in a larger sample of SCZ patients. The *DRD2*-141C *Ins/Del* polymorphism may affect its methylation rates. Methylation rates and the -141C *Ins/Del* polymorphism of *DRD2* were associated with symptoms and drug-induced extrapyramidal side effects, respectively, and may be useful biomarkers for SCZ.

Declaration of Competing Interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Acknowledgments

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Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.psychres.2019.06.001.

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