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Functional analyses and effect of DNA methylation on the *EGR1* gene in patients with schizophrenia

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ABSTRACT

EGR1, involved in the regulation of synaptic plasticity, learning, and memory, is considered a candidate gene for schizophrenia. We resequenced the exonic regions of *EGR1* in 516 patients with schizophrenia and conducted a reporter gene assay. We found two mutations including a rare mutation (c.-8C>T, rs561524195) and one common SNP (c.308-42C>T, rs11743810). The reporter gene assay showed c.-8C>T mutant did not affect promoter activity. Gene expression analyses showed that the average *EGR1* mRNA and protein levels in lymphoblastoid cell lines of schizophrenia in male, but not female, were significantly higher than those in controls. We conducted *in vitro* DNA methylation reaction, luciferase activity assay, and pyrosequencing to assess DNA methylation of *EGR1* expression underlying the pathophysiology of schizophrenia. DNA methylation of the *EGR1* promoter region attenuated reporter activity, suggesting that DNA methylation regulates *EGR1* expression. There were no statistically significant differences in DNA methylation levels of 17 CpG sites at the *EGR1* promoter region between 64 patients with schizophrenia compared with 64 controls. These results suggest that the exonic mutations in *EGR1* and DNA methylation regulating *EGR1* expression might not be associated with schizophrenia. However, the gender-specific association of elevated *EGR1* expression might be involved in the pathophysiology of schizophrenia.

1. Introduction

Schizophrenia is a severe and chronic mental disorder with a prevalence rate of roughly 1% in the general population. Schizophrenia is characterized by separation from reality with delusion formation, hallucinations, disorganized behavior, and impairment of cognitive functions (Freedman, 2003). Although twin studies indicate a substantial genetic contribution to the pathophysiology of schizophrenia (Sullivan et al., 2003), epigenetic factors account for an alternative explanation in the non-hereditary portion of schizophrenia (Shorter and Miller, 2015; Tsuang et al., 2001). Despite recent advances in genomic and epigenetic technology, the exact mechanism underlying schizophrenia remains mostly unknown.

The early growth response 1 (*EGR1*) gene encoding the immediately early protein, belongs to the EGR family of Cys_2–His_2-type zinc-finger proteins (Sukhatme, 1991). The *EGR1* gene is implicated in cell proliferation, female reproduction, immune response, cell growth, neuronal plasticity, and memory formation (Poirier et al., 2008; Veyrac et al., 2014). Notably, EGR1 is a nuclear protein and functions as a transcriptional regulator in neuronal development (O'Donovan et al., 1999). Qin showed that the *Egr1* gene is involved in N-methyl-D-aspartate receptor-mediated PSD-95 downregulation and α-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid (AMPA) receptor trafficking (Qin et al., 2015). Animal studies have shown that the *Egr1* gene is impacted by brain function, cognitive aging, and antipsychotic drug administration (Cheng et al., 2008; Penner et al., 2016). Several lines of evidence have demonstrated a decrease in *EGR1* expression in patients with schizophrenia (Liu et al., 2017; Perez-Santiago et al., 2012; Ramaker et al., 2017; Xu et al., 2016; Yamada., 2007). For example, reverse transcription quantitative PCR (RT-qPCR) analysis revealed that *EGR1* expression was significantly downregulated in peripheral blood mononuclear cells and the prefrontal cortex in patients with schizophrenia compared with controls (Liu et al., 2017; Perez-Santiago et al., 2012; Xu et al., 2016; Yamada et al., 2007). Ramaker and colleagues identified significantly downregulated expression of the *EGR1* gene in the anterior cingulate cortex of schizophrenia samples compared with

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controls (Ramaker et al., 2017). These findings indicate that the *EGR1* gene involved in the pathophysiology of schizophrenia. However, another expression study demonstrated up-regulation of the *EGR1* gene in schizophrenia fibroblasts and peripheral blood cells (Cattane et al., 2015). These inconsistent results can be explained by the heterogeneity of schizophrenia, medications, and epigenetic processes (Thomas, 2017).

Here, we examined whether there are pathologic variants of the *EGR1* gene associated with schizophrenia and whether the expression of the *EGR1* gene is subjected to epigenetic regulation in schizophrenia. To address these issues, we systemically searched for genetic variants in the exon region and intron–exon boundary region of the *EGR1* gene in 516 schizophrenic patients from Taiwan. Further, we conducted RT-qPCR and immunoblotting analysis to compare *EGR1* gene expression in the lymphoblastoid cells of patients with schizophrenia and control subjects. Additionally, we conducted an *in vitro* DNA methylation reaction, a Lucia luciferase activity assay, and pyrosequencing to assess the DNA methylation regulating the expression of the *EGR1* gene underlying the pathophysiology of schizophrenia.

2. Methods

2.1. Subjects

Patients fulfilling the diagnostic criteria of schizophrenia defined by the Diagnostic and Statistical Manual of Mental Disorders-Fourth Edition Tex Revision (DSM-IV TR) were recruited into this study. We excluded cases that had been found to be organic brain syndrome, mental retardation, substance-related psychosis, or mood disorder with psychotic features. Healthy controls were recruited from a medical center as usual medical check in east Taiwan. All subjects are Han Chinese from Taiwan. We recruited 516 patients with schizophrenia (274 males, mean age of 46 ± 11 years; and 242 females, mean age of 51 ± 11 years) for gene resequencing. We selected 64 patients with schizophrenia (37 male and 27 female participants, with an average age of 49.47 ± 9.18) and 64 controls (30 male and 34 female participants, with an average age of 49.13 ± 16.94) for gene expression and pyrosequencing analysis. The study was approved by the Antai-Tian-Sheng Memorial Hospital Institution Review Board (Approval number: 13-060-A2), and written informed consent was obtained after the procedures were fully explained. Genomic DNA was prepared from peripheral blood cells according to standard protocols and stored at –80 °C until use.

2.2. Mutation detection

Optimal PCR primer sequences were generated to amplify each exon and a flanking intron of the *EGR1* gene using the Primer3 website (http://frodo.wi.mit.edu/primer3/). The primer sequences, optimal annealing temperatures, and size of each amplicon are available on request. In the standard reaction, genomic DNA (75 ng) was amplified in a reaction volume of 15 μL containing 1 μM each of sense and antisense primer, 0.2 mM of dNTP, 50 mM of KCl, 1.5 mM of $MgCl_2$, 0.1% vol/vol of Triton X-100, 10 mM of Tris-HCl (pH 9.0), and 0.75 U Taq polymerase. PCR cycling conditions consisted of an initial denaturation at 95 °C for 5 min, followed by 30 cycles at 95 °C for 1 min, the optimal annealing temperature of each exon for 1 min, and 72 °C for 1 min. After PCR amplification, aliquots of the PCR products were processed using an Illustra™ ExoProStar™ 1-Step Kit (GE Healthcare Bio-Sciences Corp., NJ, USA) to remove residual primers and dNTPs following the manufacturer's protocol. The purified PCR products were subjected to direct sequencing using ABI Prism™ BigDye™ Terminator Cycle Sequencing Ready Reaction Kit Version 3.1 and ABI autosequencer 3730 (Perkin Elmer Applied Biosystems, Foster City, CA) according to the manufacturer's protocol. Repeated PCR and sequencing in both directions confirmed the authenticity of mutations identified in this study. Taiwan Biobank (https://taiwanview.twbiobank.org.tw) is a nationwide research database that collected, stored, and analyzed the biological data necessary for the research designed to trace the common chronic diseases occurring locally in Taiwan (Fan et al., 2008). We explored whether the mutations we identified were documented in Taiwan BioBank.

2.3. Reporter gene activity assay

Genomic DNA from the subjects was used to construct the inserts for the reporter gene activity assay. For functional characterization of c.-8C > T, sense primer (5′-tatggccatgtacgtcacga-3′) and antisense primer (5′-gaaacccggctctcattcta-3′) were used to obtain an amplicon containing identified genetic variants. The PCR fragments were cloned into pCR-Blunt II vector (Invitrogen, Carlsbad, CA, USA) and then subcloned into the pGL3-enhancer vector (Promega, Madison, WI, USA) using XhoI and HindIII recognition sites, and the authenticity of each construct was verified by sequencing. The SK-N-SH neuroblastoma cells were cultured in 96-well plates at 3000 cells per well in minimum essential media supplemented with 1 mM sodium pyruvate, 0.1 mM non-essential amino acids, penicillin–streptomycin (Invitrogen, Carlsbad, CA, USA), and 10% fetal bovine serum. The cells were co-transfected with 200 ng of reporter plasmid and 10 ng of pRL-TK (Promega, Madison, WI, USA) as an internal control reporter using Lipofectamine™ 3000 (Invitrogen, Carlsbad, CA, USA), and six replicates were performed for each treatment. At 30 h after transfection, cells were lysed, and the luciferase activities were measured using the dual-luciferase reporter assay system according to the manufacturer's instructions (Promega, Madison, WI, USA). The firefly luciferase activity was normalized against the Renilla luciferase activity in each transfection.

2.4. Lymphoblastoid cell lines, total RNA, and protein preparation

Lymphoblastoid cell lines from each subject were established by transforming lymphocytes with Epstein-Barr virus following the procedures described elsewhere (Huang et al., 2004). Total RNA of cells was purified using TRIzol reagent according to the manufacturer's standard protocol (Invitrogen Life Technologies, Carlsbad, CA, USA) and stored at –80 °C until use. For protein preparation, cells were washed twice with cold PBS and resuspended in the lysis solution containing 100 mM Tris-HCl (pH 9.0), 100 mM NaCl, 0.5% (v/v) Triton X-100, and a protease inhibitor cocktail tablet (one complete, mini tablet/10 mL, Roche Diagnostics GmbH, Mannheim, Germany). The homogenates were centrifuged at 13,000 rpm for 30 min at 4 °C, and the supernatants were stored at –80 °C until use.

2.5. RT-qPCR

cDNA was prepared by reverse transcription using SuperScript III RNase H- Reverse Transcriptase (Invitrogen) according to the manufacturer's standard protocol. The primer sequences for PCR amplification are 5′-GACCGCAGAGTCTTTTCCTG-3′ and 5′-AGCGGCCAGTATAGGTGATG-3′. The relative standard curve method was used for the quantification of *EGR1* mRNA expression. The RT-qPCR was performed using an Applied Biosystems PRISM7900 Sequence Detection System. The 18S rRNA was used as the endogenous gene for normalization. The details of RT-qPCR have been described elsewhere (Huang et al., 2004). All PCR experiments were performed in triplicate.

2.6. Immunoblotting analysis

Immunoblotting analysis was performed using standard protocols with rabbit anti-EGR1 antibody (sc-110, Santa Cruz Biotechnology, Inc., Santa Cruz, California). A horseradish peroxidase-conjugated donkey anti-rabbit IgG (NA934, Amersham) was used as a second antibody. The chemiluminescence signal was visualized using the ECL

detection system (GE Health Care Bio-Sciences AB, Uppsala, Sweden). Band intensities were assessed using NIH ImageJ software (http://rsb.info.nih.gov/nih-image/) and normalized to the intensity of the amido black staining. The differences in protein level between two groups were evaluated using Student's *t*-test. Significant differences were defined as those with $p < 0.05$.

2.7. In vitro DNA methylation reaction and Lucia luciferase activity assay

The *EGR1* putative promoter region was amplified from genomic DNA using the following primers: 5′-CATAGTACTAGGATCCGGGACTAGGGAACAGCCTTT-3′/5′-GTTACATGTTGGATCCCTCCTAGGCGTGAACTAGGC-3′ (promoter1) and 5′-CATAGTACTAGGATCCGACCCGGAAATGCCATATAA-3′/5′-GTTACATGTTGGATCCGCCTCTATTTGAAGGGTCTGG-3′ (promoter2). The PCR product was cloned into a pCpGfree-basic-Lucia vector (InvivoGen, San Diego, CA, USA), containing a Lucia luciferase reporter gene, using an In-Fusion® HD cloning kit. For methylation reactions, 2 µg plasmid was mixed with 4 U CpG methyltransferase (M.SssI; New England Biolabs), in 50 nM NaCl, 10 mM Tris-HCl pH 7.9, 10 mM MgCl2, 1 mM dithiothreitol, and S-adenosylmethionine (SAM) for 4 h at 37 °C. Methylation was measured by digesting the reaction mix with the CpG methylation-blocked enzyme. To test the effect of methylation on promoter activity, cells were co-transfected with 200 ng pCpGfree-basic-Lucia plasmid and 10 ng firefly luciferase reporter vector, the pGL3-control plasmid (Promega, Madison, WI, USA). Twenty-four hours post-transfection, luciferase activity was measured with a dual-luciferase reporter assay system. Lucia luciferase activity was normalized against firefly luciferase activity in each transfection. Each plasmid was transfected six times for each reporter gene experiment.

2.8. Bisulfite treatment of genomic DNA and pyrosequencing assay

The details of the pyrosequencing procedure were described in our previous report (Chuang et al., 2016). In brief, genomic DNA was treated with bisulfite and purified using an EpiTech Fast DNA Bisulfite kit (QIAGEN). Regions of interest were amplified by PCR and sequenced using a PyroMark Q96 ID instrument (QIAGEN). The primers for PCR amplification (5′-TGGGAGGAGGGAAGAAGG-3′ and 5′-ACACCTCCATCCTACACC-3′) and pyrosequencing (5′-GGTTAGAGTTTTAGGTTTTT-3′) were designed using PyroMark Assay Design 2.0 software (QIAGEN). PCR amplifications were performed using a PyroMark PCR kit (QIAGEN), and all PCR products were checked on a 2% agarose gel to ensure successful amplification and specificity before proceeding with pyrosequencing. The differences in DNA methylation levels of a CpG site between schizophrenic patients and the controls were assessed using Mann-Whitney-Wilcoxon test (two-tailed) with Bonferroni multiple testing. Significant differences were defined as those with $p < 0.05/17$.

3. Results

3.1. Mutation detection of EGR1 in schizophrenia and reporter gene activity assay

The human *EGR1* gene comprises two exons that span approximately 3.8 kb on chromosome 5q31.2. After resequencing of the *EGR1* gene, we found two mutations including a rare mutation (c.−8C>T, rs561524195) and one common single-nucleotide polymorphism (SNP) (c.308-42C>T, rs11743810) in 516 patients with schizophrenia (Fig. 1A). The c.-8C>T mutant was not observed in 1517 healthy controls from Taiwan BioBank. Computer analysis showed that the c.-8C>T created transcription factor binding sites for TFII-I, C/EBPbeta,

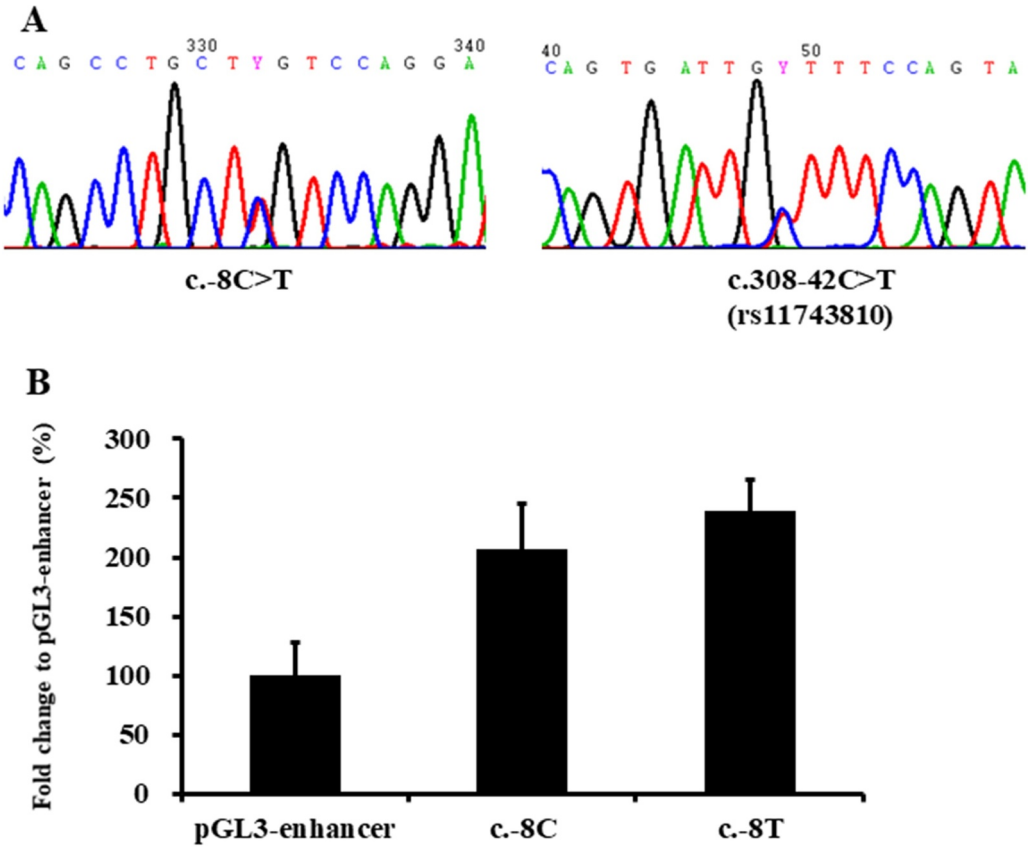

Fig. 1. Reporter gene activity assay. (A) Sequence electropherograms of the two mutations of the *EGR1* gene identified in schizophrenic patients. (B) Reporter gene activity assay of the mutant-type (c.−8T) compared with the wild-type construct (c.-8C). $n = 6$.

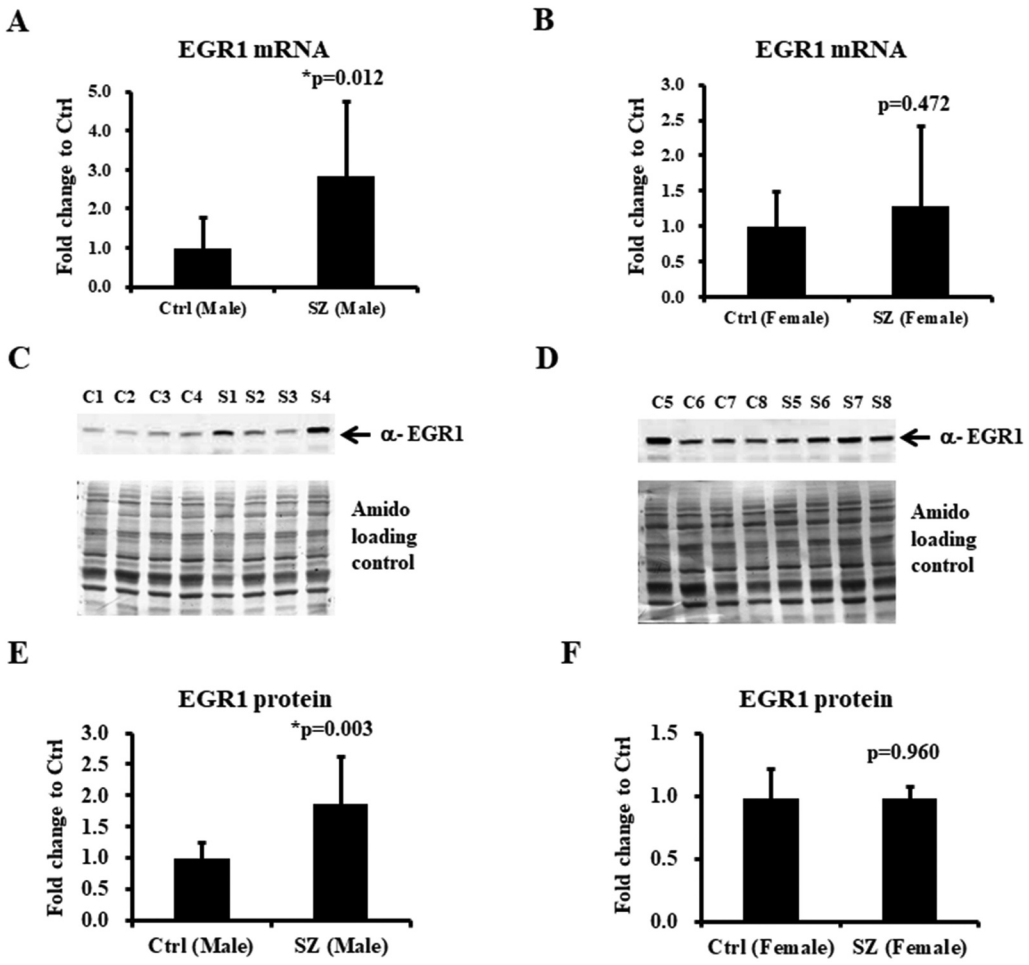

Fig. 2. *EGR1* gene expression assay. (A) Bar chart of the relative expression of *EGR1* mRNA levels in 10 male schizophrenic patients and nine male controls. (B) Bar chart of the relative expression of *EGR1* mRNA levels in nine female schizophrenic patients and 10 female controls. (C) Representative immunoblots of the *EGR1* protein in lymphoblastoid cells from male controls (C1 to C4) and male schizophrenic patients (S1 to S4). (D) Representative immunoblots of the *EGR1* protein in lymphoblastoid cells of female controls (C5 to C8) and female schizophrenic patients (S5 to S8). (E) Quantification of the band intensity of the *EGR1* protein expression between nine male schizophrenic patients and 10 male controls. (F) Quantification of the band intensity of the *EGR1* protein expression between nine female schizophrenic patients and 10 female controls. SZ = schizophrenic patient; Ctrl = control; data are expressed as fold change to control ± standard deviation (* $p < 0.05$).

and AR (Messeguer et al., 2002). We performed a reporter gene activity assay to assess the potential regulatory impact of the rare mutation c.-8C>T on the expression of the *EGR1* gene. There was no difference in the promoter activity between mutant type and wild type in SK-N-SH cells (Fig. 1B).

3.2. RT-qPCR and immunoblotting analysis

Using RT-qPCR, we compared the *EGR1* mRNA levels in lymphoblastoid cell lines between schizophrenic patients and controls. Male, but not female, patients with schizophrenia had ~2.5-fold higher *EGR1* mRNA levels than controls ($p = 0.012$, Fig. 2A and B). Using immunoblotting analysis, we compared the *EGR1* protein levels in lymphoblastoid cell lines between schizophrenic patients and controls. Male but not female patients with schizophrenia had ~2-fold higher *EGR1* protein levels than controls ($p = 0.003$, Fig. 2C–F).

3.3. Methylation of the EGR1 promoter in vitro attenuates reporter activity

We searched the potential regulatory regions of the *EGR1* gene using *in vitro* reporter gene activity assay. Two constructs with the possible regulatory elements (Promoter1: chr5:138464525–138464964; Promoter2: chr5:138465099–138465512) were cloned into a pCpGfree-basic-Lucia vector, respectively (Fig. 3A). In SK-N-SH cells, the luciferase reporter gene activity of the construct containing promoter1 and promoter2 of the *EGR1* gene was ~3 ± 0.8- and ~80 ± 30-fold higher compared with the pCpGfree-basic-Lucia vector as a control, respectively (Fig. 3B).

We artificially methylated the pCpGfree-basic-Lucia with the *EGR1* promoter region *in vitro* before transfection to assess the effects of DNA methylation on Lucia luciferase reporter expression. The constructs were methylated by M.Sss I at a concentration of 1.6 mM SAM. The methylated constructs were transfected into SK-N-SH cells, and Lucia luciferase activity was measured. Methylation of CpG sites in the *EGR1* promoter2 region, but not the *EGR1* promoter1 region, inhibited the expression of Lucia luciferase (Fig. 3C).

3.4. DNA methylation status in the EGR1 promoter region

Seventeen CpG sites (CpG1 to CpG17) in the *EGR1* promoter2 region that overlapped with the CpG island (CpG:191) were analyzed for patterns of DNA methylation using pyrosequencing assay. A significantly higher DNA methylation level at CpG site 10 of the *EGR1* gene was observed in the lymphocytes of male schizophrenic patients compared with controls (Table 1). However, the significance did not endure after correction for Bonferroni multiple testing. There is no significant DNA methylation status of the other 16 CpG sites of the *EGR1* gene in male cases versus male controls or female cases versus female controls (Table 1). *In silico* analysis showed that 17 CpG sites of the *EGR1* gene harbored transcriptional factor binding sites and factors, predicted by PROMO (Table 1).

4. Discussion

4.1. Genetic analyses of the EGR1 gene in schizophrenia

There has been growing debate that the genetic contribution to individual susceptibility to schizophrenia can be caused either by

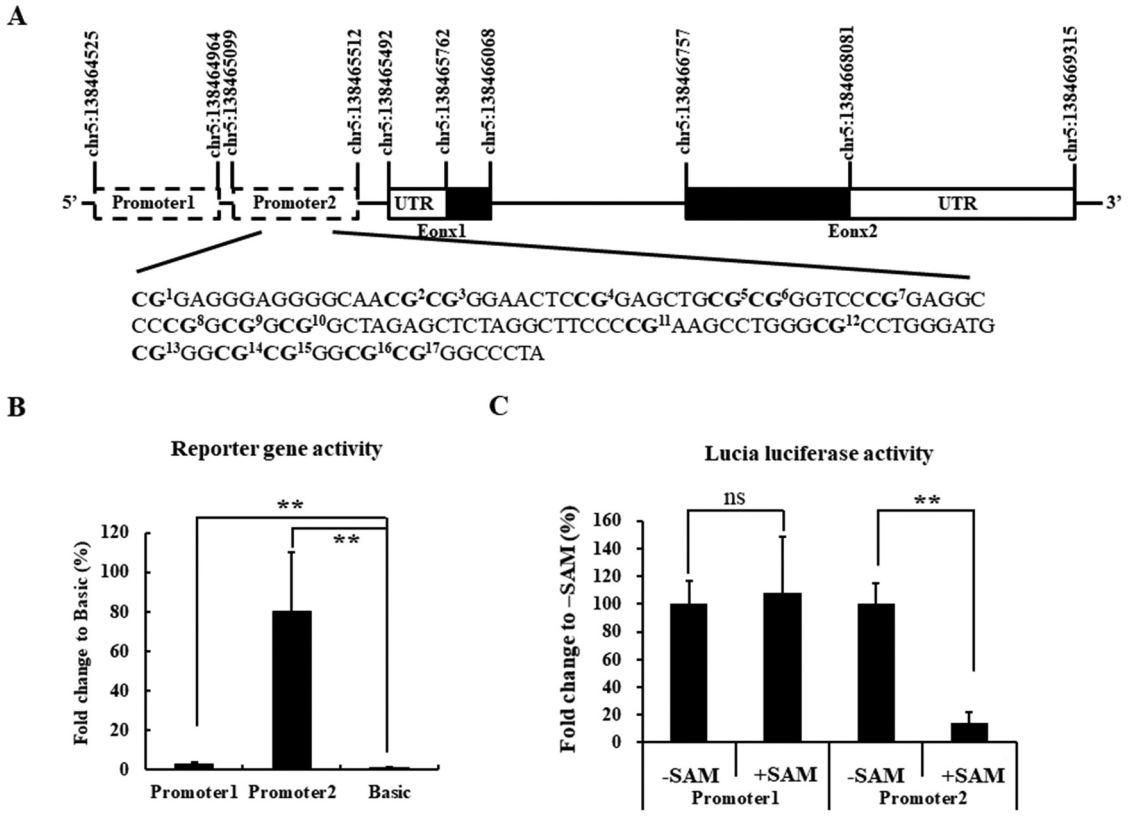

Fig. 3. Reporter gene activity assay, *in vitro* DNA methylation reaction, and Lucia luciferase activity assay. (A) A schematic representation of the locations of the two *EGR1* putative promoters and 17 CpG sites used to assess DNA methylation effects. Bold-type CG indicates the CpG site. UTR, untranslated region. (B) Reporter gene activity assay of the construct containing the *EGR1* promoter1 and the *EGR1* promoter2 compared with the pGL3-basic construct. (C) The pCpGfree-basic_EGR1-promoter2 plasmid methylated by M.Sss I and SAM inhibited the expression of Lucia luciferase in SK-N-SH cells. Values and error bars represent the mean ± standard deviation ($n = 6$, two-tailed t-test, **: $p < 0.0001$; ns, no significance).

Table 1
Transcription factor predicted binding and methylation levels of 17 CpG sites at the *EGR1* gene in schizophrenia patients (SZ) and controls (Ctrl).

CpG site	Transcription factor predicted[#]	Male SZ ($n = 37$) Mean ± SD	Male Ctrl ($n = 30$) Mean ± SD	p value[&]	Female SZ ($n = 27$) Mean ± SD	Female Ctrl ($n = 34$) Mean ± SD	p value[&]
1	GR-alpha; AP-2alphaA; TFII-I; E2F-1; MAZ	2.79 ± 2.15	2.80 ± 1.16	0.568	2.17 ± 1.92	1.78 ± 1.75	0.409
2	FOXP3; Pax-5; p53; E2F-1; RelA	8.03 ± 14.31	7.77 ± 11.00	0.749	4.87 ± 6.41	7.99 ± 16.14	0.435
3	c-Ets-1; E2F-1; RAR-beta:RXR-alpha; RelA	5.88 ± 13.39	2.25 ± 2.65	0.412	2.37 ± 2.79	4.02 ± 7.98	0.875
4	TFII-I; c-Myb; RAR-beta:RXR-alpha; RelA	10.11 ± 12.33	7.76 ± 5.20	0.831	11.26 ± 19.79	6.82 ± 7.04	0.549
5	GCF; E2F-1	7.18 ± 7.80	5.37 ± 7.77	0.055	3.50 ± 3.16	6.73 ± 11.55	0.327
6	GCF; E2F-1; RXR-alpha	3.73 ± 7.46	5.67 ± 11.99	0.148	6.83 ± 10.53	5.62 ± 12.17	0.387
7	GR-alpha; AP-2alphaA	10.97 ± 20.16	5.90 ± 4.49	0.953	4.20 ± 3.32	8.19 ± 16.16	0.705
8	ETF	15.63 ± 18.75	13.98 ± 18.18	0.175	7.87 ± 6.26	13.46 ± 23.62	0.967
9	ETF; E2F-1	7.09 ± 11.08	8.42 ± 12.29	0.742	2.25 ± 3.13	4.25 ± 6.35	0.143
10	ETF; E2F-1	6.06 ± 9.02	1.52 ± 2.93	0.037	1.61 ± 5.88	3.36 ± 8.04	0.123
11	c-Ets-1; Elk-1	4.54 ± 1.85	6.73 ± 7.67	0.088	9.82 ± 11.64	7.16 ± 10.11	0.202
12	AP-2alphaA; Pax-5; p53; RAR-beta:RXR-alpha	13.60 ± 16.39	9.83 ± 8.79	0.694	16.51 ± 18.19	12.34 ± 11.98	0.836
13	Pax-5; p53; XBP-1; E2F-1; WT1; PEA3	7.99 ± 11.13	7.57 ± 4.38	0.379	17.63 ± 15.46	10.24 ± 11.68	0.135
14	GCF; Pax-5; p53; E2F-1; WT1	8.57 ± 12.80	8.97 ± 11.98	0.592	14.43 ± 18.77	8.23 ± 10.97	0.153
15	GCF; Pax-5; p53; E2F-1; WT1	10.67 ± 13.11	8.52 ± 12.70	0.645	9.60 ± 13.22	8.41 ± 12.17	0.745
16	GCF; Pax-5; p53; E2F-1; WT1	2.90 ± 3.05	3.24 ± 4.63	0.712	6.52 ± 9.03	6.03 ± 8.29	0.477
17	GCF; Pax-5; p53; E2F-1; WT1	5.07 ± 9.88	11.32 ± 12.24	0.082	9.54 ± 21.60	5.91 ± 8.97	0.775

[&] Statistical differences between groups were analyzed using Mann–Whitney-Wilcoxon test (two-tailed). Statistical significance $p < 0.0029$ (0.05 divided by 17), Bonferroni multiple testing.

[#] Transcription factors predicted by the PROMO website.

common variants with low penetrance or by rare variants with high penetrance (Cichon et al., 2009; Gratten, 2016). Several genetic association studies have shown that the *EGR1* gene is not associated with schizophrenia, suggesting that the common SNPs of the *EGR1* gene appear not to play a significant role in conferring susceptibility to schizophrenia (Cheng et al., 2012; Liu et al., 2010; Yamada et al., 2007).

Here, we used the exonic resequencing strategy to search for rare variants in the *EGR1* gene. After resequencing, we identified two mutations (c.-8C>T, rs561524195 and c.308-42C>T, rs11743810) in 516 patients with schizophrenia. The rs561524195 was reported in the Exome Aggregation Consortium with the allele frequency of 0.00001 and was absent in 1517 healthy controls from Taiwan BioBank, suggesting that this mutation is a very rare schizophrenia-associated

mutant. *In silico* analysis predicted that this mutant creates transcription factor binding sites for TFII-I, C/EBPbeta, and AR. We presumed that this rare mutant might affect *EGR1* expression via trans-acting genetic modifiers. However, the promoter assay denied the speculation. Taken together, no rare pathologic mutations were identified in the exonic regions of the *EGR1* gene in this study.

4.2. Gender-specific association of elevated EGR1 expression in schizophrenia

Several studies have shown that there are sex differences in the brain development, gene expression, and epigenomic profile of schizophrenia (Abel et al., 2010; Chase et al., 2015). Groger and colleagues detected sex-specific stress sensitivity for the Egr1 protein. They obtained evidence for a protective effect on the postnatal stress-induced downregulation of Egr1 expression in male rats (Groger et al., 2016). Stack and colleagues found that sexually dimorphic expression patterns of Egr1 in the medial prefrontal cortex of rats can mediate sex differences in anxiety-like behavior (Stack et al., 2010). According to the above animal studies, we can infer that the transcription factor, Egr1, has a functional role in mediating sex differences in various behavior tests. Interestingly, we found that *EGR1* mRNA and protein levels in lymphoblastoid cells were significantly increased in male patients compared to male controls. Taken together, the above evidence may partly explain that the gender-specific association of elevated *EGR1* expression might be involved in the pathophysiology of schizophrenia.

4.3. The role of DNA methylation of the EGR1 gene in schizophrenia

Accumulating evidence suggests that epigenetic alterations may provide an alternative explanation for the pathogenesis of schizophrenia (Diwadkar et al., 2014; Gavin and Floreani, 2014; Ibi and Gonzalez-Maeso, 2015). We showed that the two constructs with sequences upstream of the *EGR1* gene had promoter activity, suggesting that these loci have a potential regulatory effect on *EGR1* expression. Penner et al. found that decreased *Egr1* gene expression in the aging hippocampus was associated with DNA methylation changes in the promoter region of the Egr1 gene (Penner et al., 2016). Here, we used *in vitro* DNA methylation assay to demonstrate that a promoter region of the *EGR1* gene may be regulated by DNA methylation in SK-N-SH cells. Alterations in *EGR1* expression could be derived from epigenetic DNA methylation. Thus, we hypothesized that the potential regulatory region of the *EGR1* gene containing CpG islands represented a potential target for DNA methylation and that a change in the *EGR1* methylation level may contribute to the pathophysiology of schizophrenia. We further compared the DNA methylation level of the 17 CpG sites from chr5:138465099 to 138465512 of the *EGR1* gene in the peripheral blood cells between patients with schizophrenia and healthy controls but found no difference in the DNA methylation level of the *EGR1* gene between the two groups. The explanation of the negative results is that other epigenetic mechanisms, such as posttranslational modifications, play alternative roles in *EGR1* expression and the pathophysiology of schizophrenia (Thomas, 2017; Xie et al., 2013).

4.4. Strengths and limitations

The strength of this study is the combination of functional genetic strategy, *in vitro* DNA methylation reaction, and pyrosequencing assay to investigate whether the expression of the *EGR1* gene is subjected to DNA methylation regulation in schizophrenia. The major limitation of this study is the small sample size in RT-qPCR, immunoblotting, and DNA methylation experiments. Therefore, the present results can be considered as only preliminary. Further replication studies with larger sample sizes are warranted.

4.5. Concluding remarks

We found no evidence to suggest that the exonic mutations in the *EGR1* gene and DNA methylation regulating the expression of the *EGR1* gene are associated with schizophrenia. However, we found that elevated *EGR1* expression might be associated with the pathophysiology of schizophrenia in males.

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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.2019.03.044.

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