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Genetic and functional analyses of early growth response (EGR) family genes in schizophrenia

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ABSTRACT

Objective: Early growth response genes (*EGR1*, *2*, *3*, and *4*) encode a family of nuclear proteins that function as transcriptional regulators. They are involved in the regulation of synaptic plasticity, learning, and memory, and are implicated in the pathogenesis of schizophrenia.
Methods: We conducted a genetic association analysis of 14 SNPs selected from the *EGR1*, *2*, *3*, and *4* genes of 564 patients with schizophrenia and 564 control subjects. We also conducted Western blot analysis and promoter activity assay to characterize the EGR genes associated with schizophrenia
Results: We did not detect a true genetic association of these 14 SNPs with schizophrenia in this sample. However, we observed a nominal over-representation of C/C genotype of rs9990 of *EGR2* in female schizophrenia as compared to female control subjects ($p = 0.012$, uncorrected for multiple testing). Further study showed that the average mRNA level of the *EGR2* gene in the lymphoblastoid cell lines of female schizophrenia patients was significantly higher than that in female control subjects ($p = 0.002$). We also detected a nominal association of 4 SNPs (rs6747506, rs6718289, rs2229294, and rs3813226) of the *EGR4* gene that form strong linkage disequilibrium with schizophrenia in males. Reporter gene assay showed that the haplotype T-A derived from rs6747506 and rs6718289 at the promoter region had significantly reduced promoter activity compared with the haplotype A-G.
Conclusion: Our data suggest a tendency of gender-specific association of *EGR2* and *EGR4* in schizophrenia, with an elevated expression of *EGR2* in lympoblastoid cell lines of female schizophrenia patients and a reduced *EGR4* gene expression in male schizophrenia patients.
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1. Introduction

Schizophrenia is a severe chronic mental illness characterized by abnormal perceptions, thought disturbances, bizarre behaviors, and impaired cognitive functions. This disease affects approximately 1% of the general population worldwide. The etiology and pathogenesis of schizophrenia are still unclear today, but studies have shown that schizophrenia might result from complex interaction between genetic and environmental factors. The estimated heritability of schizophrenia is approximately 80% on average, suggesting genetic factors play a major role in the pathogenesis of schizophrenia (Robertson et al., 2006).

The early growth response (EGR) gene family encodes inducible transcription factors that are involved in the regulation of several biological processes, such as cell differentiation, mitogenesis, biological rhythm, and muscle development (Gitenay and Baron, 2009; Madden and Rauscher, 1993; O'Donovan et al., 1999; Sukhatme, 1990, 1991). The EGR family comprises four members: *EGR1*, *EGR2*, *EGR3*, and *EGR4*; they share a common DNA binding domain composed of three zinc-finger motifs at their C-terminal (O'Donovan et al., 1999). Recently, several studies suggest that the EGR family is involved in the molecular mechanism of synaptic plasticity related to learning and memory (Alberini, 2009; Poirier et al., 2008). Increased expression of *Egr1* has been reported as being associated with the formation

Abbreviations: COMT, catechol-O-methyltransferase; DSM-IV, diagnostic and statistical manual of mental disorders-IV; EGR, early growth response; HRM, high resolution melting; MAF, minor allele frequency; NF-kappaB, nuclear mediator nuclear factor-kappa B; NMDAR, N-methyl-D-aspartate receptor; LD, linkage disequilibrium; LTD, long-term depression; LTP, long-term potentiation; PCP, phencyclidine; RFLP, restriction fragment length polymorphism; SNP, single nucleotide polymorphism.

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of emotional memory (Baumgartel et al., 2008), while *Egr1* knockout mice showed impairments in late long-term potentiation (LTP) and long-term memory (Jones et al., 2001). One study reported that *Egr3* knockout mice showed deficits in both early- and late-phase hippocampal LTP, which led to impaired short-term and long-term hippocampus- and amygdala-dependent learning and memory (Li et al., 2007). Another study reported that *Egr3* knockout mice displayed accentuated behavioral responses to mild stress, an abnormal response to novelty, and defects in immediate memory, which were attributed to a failure to establish hippocampal long-term depression (LTD) (Gallitano-Mendel et al., 2007). It was surprising that the *Egr2*-deficient mice did not show impairment in learning and memory, but showed improved performance in motor learning and object memory (Poirier et al., 2007).

In a genetic study of calcineurin pathway in schizophrenia in a Japanese sample, Yamada and colleagues reported a nominal association of SNPs of *EGR1*, *EGR2*, and *EGR3* genes with schizophrenia (Yamada et al., 2007). They also found reduced *EGR1*, *EGR2*, and *EGR3* transcripts in the dorsolateral prefrontal cortex of the postmortem brain in patients with schizophrenia. In a further study with a larger-sized sample of subjects, they found a statistically significant association of *EGR3* gene with schizophrenia, indicating that the EGR gene family might be involved in the pathogenesis of schizophrenia (Yamada et al., 2007). Moreover, antipsychotic drugs such as haloperidol, clozapine, risperidone, and aripiprazole have been shown to induce differential expression of the *Egr* family genes in rat brain (Cheng et al., 2008; Nguyen et al., 1992; Verma et al., 2006, 2007). In our previous study, we reported that chronic administration of a new class of antipsychotic drug, aripiprazole, induced expression of mRNA of *Egr1*, *Egr2*, and *Egr4* in the frontal cortex of rats, and that this might be related to its clinical efficacy (Cheng et al., 2008). Prompted by these findings, we were interested to know whether the EGR family genes are associated with schizophrenia in our population.

2. Materials and methods

2.1. Subjects

All subjects were Han Chinese from Taiwan. Patients fulfilling the diagnostic criteria for schizophrenia defined by the Diagnostic and Statistical Manual of Mental Disorders-IV (DSM-IV) were recruited into this study. The diagnosis of schizophrenia was based on clinical interviews and reviews of medical records by senior psychiatrists with consensus. Exclusion criteria included psychosis due to a general medical condition, substance-related psychosis, and mood disorder with psychotic features. Control subjects were recruited from those who received routine medical checkups from the department of family Medicine of a general hospital in eastern Taiwan. The mental status and history of mental illness of the control subjects were evaluated by a senior psychiatrist; subjects diagnosed with a DSM-IV axis I disorder were excluded. The study was approved by the institutional review board of each participating hospital, and written informed consent was obtained after the procedures were fully explained. The patient group comprised 564 schizophrenia patients (282 males, mean age $=44\pm13$ years; and 282 females, mean age $=45\pm12$ years), and the control group was composed of 564 subjects (282 males, mean age $=43\pm15$ years; and 282 females, mean age $=45\pm13$ years). Genomic DNA was prepared from peripheral blood cells according to standard protocols.

2.2. SNP selection and genotyping

A total of 14 SNPs from 4 EGR genes were selected from the dbSNP of the National Center for Biotechnology Information (http://www.ncbi.nlm.nih.gov/), mainly based on the study published by Yamada et al. (2007). Except for rs10995315 of the *EGR2*, rs35201266 of the *EGR3*, and rs2229294 of the *EGR4*, the genotyping of the other SNPs was performed by the Sequenom MassARRAY iPLEX platform at the National Genotyping Center of Taiwan according to the standard protocol (Sequenom, San Diego, CA, USA). The genotyping of rs10995315 of the *EGR2* was performed by PCR-based direct sequencing using an ABI Prism™ BigDye™ Terminator Cycle Sequencing Ready Reaction Kit Version 3.1, and an ABI autosequencer 3730 (Perkin Elmer Applied Biosystems, Foster City, CA), according to standard protocol, and the genotyping of rs35201266 of the *EGR3* and rs2229294 of the *EGR4* was conducted using PCR-based restriction fragment length polymorphism (RFLP) analysis.

For the genotyping of rs35201266 of *EGR3*, aliquots of PCR products were digested with 1 unit of AvaII overnight at 37 °C incubation, and then subjected to 2% agarose gel electrophoresis. The A allele yielded 116 and 104 bp PCR fragments, and the G allele remained uncut (220 bp). For genotyping of rs3813226 of the *EGR4*, aliquots of PCR products were digested with 1 unit of FokI overnight at 37 °C incubation, and then subjected to 2% agarose gel electrophoresis. The G allele yielded 196 and 114 bp PCR fragments, while the A allele remained uncut (310 bp).

The genotyping of rs6718289 of the *EGR4* was conducted using high resolution melting (HRM) analysis. In brief, genomic DNA (25 ng) was amplified in a reaction volume of 10 µl containing 0.25 µM of three primers (Allele G primer with 6-bp GC tail: 5′-GCGGGCCAAGAAATG-AATGGCAATAATCTCTG-3′; Allele A primer with 14 bp GC: 5′-GCGGGC-AGGGCGGCAAGAAATGAATGGCAATAATCTCTA-3′; and reverse common primer: 5′-TGAGAGCACATAAATGTCCAGGC-3′), LCGreen PreMix2.0 (Top Bio Co., Taiwan). PCR cycling conditions consisted of an initial denaturation at 95 °C for 2 min, followed by 45 cycles at 94 °C for 30 s, and 70 °C for 30 s. PCR was performed with a DNA Engine Dyad (MJ Research, Watertown, MA); the product was then subjected to the LightScanner (Idaho Technology Inc., UT) system for high resolution melting (HRM) analysis. Before the HRM step, the PCR products were heated to 94 °C and then slowly cooled to 20 °C to promote heteroduplex formation and detection. HRM analysis was carried out within range from 65 °C to 96 °C. Genotyping scanning analyses were carried out using the LightScanner software. All primers and probes flanking the SNPs were designed using SpectroDESIGNER software (Sequenom, San Diego, CA, USA) or the Primer3 website (http://frodo.wi.mit.edu/primer3/). All the primers and probe sequences are available upon request.

2.3. Genetic association analysis

We used the SHEsis computer program to implement case–control genetic association analysis (Shi and He, 2005). The computer program uses the Monte Carlo simulation test, normal chi-square test, and odds ratio test for alleles and genotypes on single locus and multi-loci haplotypes. Deviation from Hardy–Weinberg equilibrium for each SNP was checked by a chi-square goodness-of-fit test. Pairwise linkage disequilibrium of SNPs was performed using Haploview version 4.2 (Barrett et al., 2005). Any significant p-value was corrected for multiple testing using the Bonferroni correction. Post-hoc power analysis was performed using the Genetic Power Calculator (http://pngu.mgh.harvard.edu/~purcell/gpc/) under the assumption of the following parameters: multiplicative inheritance mode, genotype relative risk $=1.2$, prevalence of disease $=0.01$, alpha level $=0.05$, and risk allele frequency of each SNP (Purcell et al., 2003). The minor allele frequency (MAF) of these SNPs ranges from 0.077 to 0.418.

2.4. Western blot analysis

Proteins were extracted from the lymphoblastoid cell lines of 64 schizophrenic patients (32 males and 32 females) and 64 normal controls (32 males and 32 females). In brief, 10^7 cells were harvested by

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