Association between EFHD2 gene polymorphisms and schizophrenia among the Han population in northern China

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Abstract
Objective: Schizophrenia is a severe neurodevelopmental disorder with a complex genetic and environmental etiology. The gene encoding EF-hand domain-containing protein D2 (EFHD2) may be a genetic risk locus for schizophrenia.

Methods: We genotyped four EFHD2 single-nucleotide polymorphisms (281 schizophrenia cases [SCZ], 321 controls) from northern Chinese Han individuals using Sanger sequencing and polymerase chain reaction-restriction fragment length polymorphism analysis. Differences existed in genotype, allele, and haplotype frequency distributions between SCZ and control groups.

Results: The rs2473357 genotype and allele frequency distributions differed between SCZ and controls; however, this difference disappeared after Bonferroni correction. Differences in rs2473357 genotype and allele frequency distributions between SCZ and controls were more pronounced in men than in women. The G allele increased schizophrenia risk (odds ratio = 1.807, 95% confidence interval = 1.164–2.803). Among six haplotypes (G–, A–, G-insC, A-C, G-C, and G-T), the G– haplotype frequency distribution differed between SCZ and controls in women; the A-C and G-C haplotype frequency distributions differed between SCZ and controls in men.

Conclusions: EFHD2 may be involved in schizophrenia. Sex differences in EFHD2 genotype and allele frequency distributions existed among schizophrenia patients. Further research is needed to determine the role of EFHD2 in schizophrenia.

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Introduction

Schizophrenia (SCZ) is a complex disease that results from a combination of genetic and environmental factors. Numerous epidemiological studies have shown that the heritability of SCZ reaches 80%, with a lifetime prevalence of 0.5% to 1.5%. It is characterized by distortions in thinking, opinion, emotion, language, self-awareness, and behavior. The most common symptoms of SCZ are hallucinations and delusions. Accumulating evidence indicates that genetic factors play an important role in SCZ, but the precise mechanisms of the disease remain unclear.

The pathophysiology of SCZ is believed to involve many neurotransmitters. Synaptic Ca\(^{2+}\) ion concentrations have an important role in neurotransmission. Previous studies have reported that Ca\(^{2+}\) dysregulation and synaptic dysfunction may be related to SCZ. EF-hand domain-containing protein D2 (EFHD2) is a Ca\(^{2+}\)-binding protein, and the gene that encodes this protein is located in the 1p36.21 locus in humans and the 5q36 locus in mice. EFHD2 plays an important role in the nervous system in mammals and is expressed similarly in all parts of the brain, including the brainstem, cerebellum, amygdala, striatum, hippocampus, cortex, and prefrontal cortex. EFHD2 regulates axonal transport, axonal generation, actin remodeling, and synaptic plasticity. Much research has been conducted to investigate the involvement of this protein in various neurological diseases. Previous studies have reported that EFHD2 is altered in Alzheimer’s disease, Parkinson’s disease, suicide, and alcohol dependence. The rs112146896 single-nucleotide polymorphism (SNP) of EFHD2 has been demonstrated to positively correlate with algebra dependence and negatively correlate with anxiety in healthy adolescents. Moreover, previous studies of SCZ patients in the United States have reported that changes in EFHD2 expression are closely related to SCZ.

The actions of Ca\(^{2+}\) are considered pivotal in the dopamine hypothesis of SCZ. Alterations in the expression of many Ca\(^{2+}\)-related proteins have been identified in SCZ, corroborating the concept that Ca\(^{2+}\) homeostasis is disrupted in this disease. Intracellular Ca\(^{2+}\) levels control dopamine receptor function and maintain neurotransmitter exocytosis during stimulation. EFHD2 is a Ca\(^{2+}\) sensor protein that exists in neuronal axons and participates in actin-mediated transport in neurons, and may be closely related to SCZ. Furthermore, synaptic dysfunction is closely related to SCZ, and EFHD2 is involved in the transport of vesicles containing synaptic proteins, and may be a synaptic protein itself. EFHD2 expression can promote synaptic development in neurons and regulate synaptic plasticity. The microtubule-associated protein (MAP) family has also been shown to be a therapeutic target for SCZ. Axonal transport requires the participation of MAP, and EFHD2 affects axonal transport through MAP.
Therefore, EFHD2 may also be involved in SCZ through its actions on MAP.

There are innate biological differences between male and female individuals. Sex differences have been found in brain function and the susceptibility to neurological diseases, as well as in the incidence, manifestations, and treatment methods of neurological diseases. For example, the risk of Parkinson’s disease, autism spectrum disorder, and addiction is higher in the male population. In contrast, women have a higher risk of Alzheimer’s disease and depression. Most studies have reported no sex differences in the overall prevalence of SCZ, but male SCZ patients are generally younger than female SCZ patients. Men with SCZ also exhibit more cognitive impairment and have lower temporal lobe volumes than female SCZ patients. Moreover, previous studies have revealed that sex differences in certain mental illnesses are caused not only by hormones, but also by genetic effects. We therefore investigated any possible sex differences in the involvement of EFHD2 in SCZ.

The role of EFHD2 in the etiology of SCZ remains uncertain. In the Han population in northern China, few studies have investigated the association between EFHD2 and SCZ. Therefore, the present study used Sanger sequencing and polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) analysis to study the association between EFHD2 polymorphisms and SCZ in the Han population.

Materials and Methods

Sample
The present study evaluated blood samples from SCZ and control subjects from the Han population in northern China. Blood samples from SCZ patients were provided by the Third People’s Hospital of Liaoning Province, and all patients met the diagnostic criteria of the Diagnostic and Statistical Manual of Mental Disorders, 4th edition. We excluded samples from patients with other mental illnesses (e.g., depression, Alzheimer’s disease) and from non-Han populations. Control blood samples from healthy individuals were provided by the Forensic Evidence Department of China Medical University. The results of questionnaires confirmed no history of mental disease for at least three generations in the control subjects. All of the SCZ and control subjects signed an informed consent form to participate in the study. The study was conducted in accordance with the Declaration of Helsinki. The sample collection and analysis were approved by the Ethics Committee of China Medical University, Shenyang, Liaoning, China (8 May 2019).

DNA extraction
Whole-genome DNA was extracted using the phenol–chloroform method and quantified by ultraviolet spectrophotometry.

Polymerase chain reaction amplification
Primer Premier 5.0 software (Premier Biosoft, San Francisco, CA, USA) and the PubMed Blast alignment function were used to design two pairs of specific primers (Table 1). These primers were designed to amplify the 1020 bp (−763 bp to +357 bp; with ATG as +1) fragment of the 5’ end and the 660 bp (+20380 bp to +21,040 bp; with ATG as +1) fragment of the 3’ end of the EFHD2 gene. The volume used for the PCR reaction system was 20 μL, which contained 2 μL of DNA (~60 ng), 1.5 μL of forward primer (7.5 pmol), 1.5 μL of reverse primer (7.5 pmol), 0.2 μL of rTaq DNA polymerase (1 U; Takara, Dalian, China), 10 μL of 2 × GC buffer, 2 μL of dNTP, and 2.8 μL of deionized water.
The PCR reaction conditions were as follows: 94°C for 5 minutes; 30 cycles at 94°C for 30 s, 57°C for 30 s, and 72°C for 90 s; a final extension at 72°C for 7 minutes. The PCR products were separated by 1% agarose gel electrophoresis.

**Sequence analysis of the polymerase chain reaction products**

The PCR amplification products of the 5’-end fragment of *EFHD2* were sequenced by Taihe Biotechnology Co. (Beijing, China) using Sanger sequencing technology. The sequencing primers of the 5’-end fragment were 5’-GCTATTGTGCAAGTC ATCCGTGTG-3’ (forward) and 5’-GCTGCCCCGTTCAGCCG-3’ (reverse). The sequencing results were analyzed using Chromas 2.23 (Technelysium Pty. Ltd., Brisbane, Australia) and DNAman 8.0 (Lynnon Biosoft, San Ramon, CA, USA) software. The rs2473357 and rs10927785 SNPs of the 3’ PCR amplification products were genotyped using BstSI and MspI restriction enzymes, respectively, and the RFLP technique. The volume of the reaction system was 10 μL: 1 μL (1 U) of restriction enzyme (New England Biolabs, Beijing, China), 1 μL of CutSmart buffer (New England Biolabs, Beijing, China), 1 μL of PCR product, and 7 μL of sterile deionized water. The rs2473357 locus was cut by the BstSI restriction enzyme in CutSmart buffer in a 50°C water bath. The rs10927785 locus was cut by the MspI restriction enzyme in CutSmart buffer in a 37°C water bath. The digested products were separated by 1% agarose gel electrophoresis after 1 hour in a water bath. The pattern diagram of the *EFHD2* gene is shown in Figure 1.

**Statistical analysis**

PowerStats 1.2 software (Promega, Madison, WI, USA) was used to calculate the genotype and allele frequencies, as well as the frequencies of the dominant,
recessive, codominant, and superdominant models. The Hardy–Weinberg equilibrium test and linkage disequilibrium analysis were performed using Haploview 4.0 software (Broad Institute, Cambridge, MA, USA). IBM SPSS Statistics for Windows, version 20.0 (IBM, Armonk, NY, USA) was used to calculate the odds ratios (ORs) and 95% confidence intervals (CIs). The χ² test was used to compare genotype, allele, and haplotype frequency distributions between the SCZ and control groups. The Bonferroni correction was used for multiple independent tests.

Results
We evaluated 602 blood samples from the Han population in northern China, including 281 cases of SCZ (138 female and 143 male subjects) and 321 controls (166 female and 155 male subjects). The mean age of the patients with schizophrenia was 41.1 ± 7.1 years (mean ± SD; range 21–65 years). The mean age of individuals in the control group was 43.7 ± 7.4 years (range 25–65 years). From the sequencing map, we identified four SNPs in EFHD2 in this population. The distributions of the four SNPs in the control group were in Hardy–Weinberg equilibrium. Comparisons of the genotype and allele frequency distributions of the four SNPs between the control and SCZ groups revealed that both the genotype and allele distributions of rs2473357 were significantly different between the SCZ and control groups (p = 0.046 and p = 0.019, respectively; Table 2). The G allele of rs2473357 increased the risk of SCZ (OR_G = 1.453, 95% CI = 1.062–1.987). Next, we constructed four genetic models. The results from these models indicated that the GG genotype of rs2473357 increased the risk of SCZ compared with the AA+AG genotype (OR_GG = 2.708, 95% CI = 1.098–6.682, p = 0.025). There was also a significant difference in the GG vs. AA gene model of rs2473357 between the SCZ and control groups (p = 0.019). After the Bonferroni correction, however, these differences were no longer significant. The genotype and allele distributions of the other loci were not significantly different between the SCZ and control groups. After stratification processing, there were statistically significant differences in the rs2473357 genotype and allele frequency distributions (p = 0.035 and p = 0.008, respectively) between the SCZ and control groups in the male population (Table 3), with a higher risk of SCZ in men who carried the G allele (OR_G = 1.807, 95% CI = 1.164–2.803). No such difference was found in the female population (data not shown).

Using Haploview 4.0 software, we analyzed the linkage disequilibrium of four SNPs in the SCZ and control groups. The linkage disequilibrium coefficient, D', was used to indicate the degree of linkage tightness (Figure 2). The linkage analysis showed that the rs71631726 and rs140124965 loci in EFHD2 were closely linked. In addition, the rs2473357 and rs10927785 loci in the 3' end of EFHD2 were transmitted in the same block. Therefore, two haplotype blocks were identified. Among these blocks, the rs71631726 (–413G/A) and rs140124965 (–413_–412insC) loci were in Block 1, which formed three haplotypes: G–, A–, and G-insC. The rs2473357 (20655A/G) and rs10927785 (20835C/T) loci were in Block 2, which also formed three haplotypes: A-C, G-C, and G-T. The haplotype frequency distributions of EFHD2 in the SCZ and control groups in both male and female subjects are shown in Table 4. The distribution of the G– haplotype in Block 1 was different between the SCZ and control groups in female subjects (p = 0.042), and the G– haplotype increased the risk of SCZ (OR_G– = 1.432, 95% CI = 1.013–2.024). The frequency distribution of the
A-C and G-C haplotypes were different between the SCZ and control groups in male subjects \((p = 0.026\) and \(p = 0.049\)). The A-C haplotype decreased the risk of SCZ in male subjects \((\text{OR}_{A-C} = 0.616, 95\% \text{ CI} = 0.401–0.947)\), and the G-C haplotype increased the risk of SCZ in male subjects \((\text{OR}_{G-C} = 1.625, 95\% \text{ CI} = 0.999–2.647)\).
Table 3. Distribution of genotype and allele frequencies, frequency distributions, and schizophrenia (SCZ) risk for the four single-nucleotide polymorphism loci in EFHD2 in male control and SCZ subjects.

| SNP        | Genotype     | Case | Control | p   | OR   | 95% CI       |
|------------|--------------|------|---------|-----|------|--------------|
| rs71631726 | GG           | 100  | 122     | 0.980 |      |              |
|            | GA           | 32   | 37      |      |      |              |
|            | AA           | 6    | 7       |      |      |              |
|            | A            | 44   | 51      | 0.844 | 1.045| 0.674–1.621  |
| rs140124965| /C0/C0       | 105  | 118     | 0.681 |      |              |
|            | /insC        | 32   | 47      |      |      |              |
|            | InsC/insC    | 1    | 1       |      |      |              |
|            | insC         | 34   | 49      | 0.383 | 0.811| 0.507–1.298  |
| rs2473357  | AA           | 89   | 129     | 0.035 |      |              |
|            | AG           | 42   | 33      |      |      |              |
|            | GG           | 7    | 4       |      |      |              |
|            | G            | 56   | 41      | 0.008 | 1.807| 1.164–2.803  |
| rs10927785 | CC           | 126  | 156     | 0.608 |      |              |
|            | CT           | 10   | 9       |      |      |              |
|            | TT           | 2    | 1       |      |      |              |
|            | T            | 14   | 11      | 0.277 | 1.559| 0.696–3.493  |

Abbreviations: CI, confidence interval; OR, odds ratio; SNP, single-nucleotide polymorphism.

Figure 2. Linkage disequilibrium diagram of four single-nucleotide polymorphism loci in the EFHD2 gene. The numbers are the multiallelic D', which represent the level of recombination between the two blocks. (a) schizophrenia group; (b) control group.
Discussion

The present study explored the associations between polymorphisms of the 5'- and 3'-end regulatory regions of the EFHD2 gene and SCZ in 281 SCZ patients and 321 healthy controls. The study was conducted in Han Chinese individuals from northern China. The rs71631726 (–413G/A) and rs140124965 (–413_–412insC) loci in the 5'-end regulatory region of EFHD2 were identified by Sanger sequencing, and the rs2473357 (þ20655A/G) and rs10927785 (þ20835C/T) loci in the 3'-end regulatory region were investigated by RFLP. No new mutation loci were identified.

The genotype and allele frequency distributions of the rs2473357 locus were significantly different between healthy controls and SCZ patients in our study population. The G allele of rs2473357 may increase the risk of SCZ. To further analyze this difference, we constructed four gene models. There were significant differences in the GG vs. AA gene model and the GG vs. AA þ AG gene model of rs2473357 between the SCZ and control groups, suggesting that the GG genotype may increase the risk of SCZ. After the Bonferroni correction, however, this difference disappeared. We speculate that the Bonferroni correction may increase the probability of false negatives because of the relatively small sample size. The remaining three SNPs were not significantly different.

### Table 4. Comparison of EFHD2 haplotypes between the schizophrenia and control groups.

| Haplotype block | Haplotype | SCZ (n = 562) | Control (n = 642) | p     | OR   | 95% CI      |
|-----------------|-----------|--------------|------------------|-------|------|-------------|
|                 |           | n   | %    | n   | %    |       |       |       |
| Block 1 G/—     | All       | 405 | 72.2 | 436 | 68.0 | 0.117 | 1.219 | 0.951–1.562 |
|                 | Male      | 200 | 72.7 | 238 | 71.3 | 0.832 | 1.039 | 0.728–1.484 |
|                 | Female    | 205 | 71.5 | 198 | 63.9 | 0.042 | 1.432 | 1.013–2.024 |
|                 | A/—       | 76  | 13.6 | 93  | 16.9 | 0.631 | 0.923 | 0.666–1.280 |
|                 | Male      | 41  | 15.0 | 47  | 14.2 | 0.807 | 1.058 | 0.673–1.664 |
|                 | Female    | 35  | 12.4 | 46  | 14.8 | 0.355 | 0.800 | 0.499–1.283 |
| Block 2 A/C     | All       | 459 | 81.7 | 551 | 85.9 | 0.051 | 0.736 | 0.541–1.001 |
|                 | Male      | 220 | 79.7 | 287 | 86.7 | 0.026 | 0.616 | 0.401–0.947 |
|                 | Female    | 239 | 83.5 | 264 | 85.1 | 0.592 | 0.886 | 0.569–1.379 |
|                 | G/C       | 79  | 14.1 | 67  | 10.5 | 0.055 | 1.404 | 0.992–1.987 |
|                 | Male      | 42  | 15.2 | 33  | 10.0 | 0.049 | 1.625 | 0.999–2.647 |
|                 | Female    | 37  | 13.0 | 34  | 11.1 | 0.458 | 1.206 | 0.734–1.981 |
|                 | G/T       | 23  | 4.1  | 17  | 2.7  | 0.163 | 1.569 | 0.829–2.967 |
|                 | Male      | 14  | 3.1  | 8   | 2.3  | 0.080 | 2.164 | 0.894–5.237 |
|                 | Female    | 9   | 5.1  | 9   | 3.1  | 0.862 | 1.087 | 0.425–2.775 |

Abbreviations: CI, confidence interval; OR, odds ratio; SCZ, schizophrenia.
between the SCZ and control groups. Haplotype analysis revealed that the two SNPs (rs71631726 and rs140124965) at the 5' end of EFHD2 comprised three haplotypes: G–, A–, and G-insC. The two SNPs at the 3' end (rs2473357 and rs10927785) also comprised three haplotypes: A-C, G-C, and G-T. The results showed that the frequency of the G– haplotype was significantly lower in female controls than in female SCZ patients, but the A-C haplotype was higher in male controls than in male SCZ patients, and the G-C haplotype was lower in male controls than in male SCZ patients. Previous studies have investigated the relationship between EFHD2 and neurological diseases. Ferrer-Acosta et al.\(^8\) and Tseveleki et al.\(^9\) reported that EFHD2 expression is increased in mouse models of Alzheimer's disease; however, Borger et al.\(^7\) reached the opposite conclusion. The EFHD2 protein has been reported to be altered in cases of suicide\(^11\) as well as in individuals with anxiety,\(^12\) alcohol dependence,\(^12,13\) Parkinson's disease,\(^25\) and amyotrophic lateral sclerosis.\(^26\) These mental disorders may have a similar genetic basis to SCZ.\(^27\) Martins-de-Souza et al.\(^15\) performed proteomic analyses of post-mortem prefrontal cortex samples from nine SCZ patients and seven normal controls and revealed that EFHD2 protein expression was higher in the prefrontal cortex of SCZ patients than of controls. These authors then performed a proteomic analysis to investigate glycolytic function in the thalamus and cerebrospinal fluid of 11 SCZ patients and eight normal controls, and again reported that EFHD2 expression was higher in SCZ patients than in controls.\(^16\) The rs2473357 SNP is located in the 3' end regulatory region of the EFHD2. The base sequence in this region undergoes RNA transcription without being translated into amino acids; the transcriptional products are removed during the modification of mature mRNA. Genetic variations in this region can cause various pathophysiological alterations.\(^24\) This site may therefore be a target region of transcription factors and cis-regulatory elements that can influence EFHD2 expression by regulating the expression and stability of mRNA. EFHD2 is a \(Ca^{2+}\)-binding protein that regulates \(Ca^{2+}\) ion concentrations.\(^24\) The actions of \(Ca^{2+}\) are considered pivotal in the dopamine hypothesis of SCZ,\(^17\) thus suggesting the involvement of EFHD2 in SCZ.

The pathogenesis of SCZ is associated with many risk factors, including sex.\(^28,29\) There are sex differences in brain function and susceptibility to mental illnesses, as well as in the incidence, manifestations, and treatment methods of mental illness. These differences are determined by innate physiological differences between men and women. For example, male individuals are more likely to develop Parkinson's disease, autism spectrum disorder, attention-deficit/hyperactivity disorder, and addiction, whereas female individuals are more likely to develop Alzheimer's disease, anxiety, and depression.\(^18\) Ochoa et al.\(^19\) reported that the rate of SCZ is higher in men than in women, and that women respond more favorably to SCZ treatment than men. Previous studies have also demonstrated sex differences in the distribution of the neurotransmitters dopamine and \(\gamma\)-aminobutyric acid in individuals with SCZ.\(^30\) EFHD2 plays an important role in neurotransmission, suggesting that EFHD2 expression may also show sex differences in SCZ patients. In the present study, there were significant differences in rs2473357 genotype and allele frequency distributions between the SCZ and control groups in the male subjects, suggesting that men who carry the G allele have a higher risk of SCZ. There were no such differences in genotype or allele frequency distributions between the SCZ and control groups in the female subjects.
To further explore the sex differences in EFHD2 expression in SCZ, we stratified six haplotypes by sex. We found that the distribution of the G– haplotype of the rs71631726 and rs140124965 loci was significantly different between the SCZ and control groups in the female subjects only, suggesting that the G– haplotype increases the risk of SCZ in women. We also found that the distributions of the A-C and G-C haplotypes of the rs2473357 and rs10927785 loci were significantly different between the SCZ and control groups in the male subjects, suggesting that the A-C haplotype reduces the risk of SCZ in men, while the G-C haplotype increases the risk of SCZ in men. The present results indicate that multiple SNPs may exert a combined effect on SCZ risk in the female population. Previous studies have also reported sex differences in haplotypes in SCZ. Gogos et al. reported that estrogen plays an important role in SCZ in women. SCZ onset may therefore be related to sex, but the specific mechanism by which sex affects SCZ remains to be elucidated.

Genes related to SCZ have also been reported to have racial differences. In the present study, we compared ethnic groups in the NCBI database (http://www.ncbi.nlm.nih.gov; accessed April 9 2020) and found that the allele frequency distribution of the rs2473357 (+20655A/G) locus in EFHD2 in the northern Han Chinese population was opposite to that of the African population, suggesting that the G allele of rs2473357 may have opposing influences on SCZ in different races. The present study provides information on EFHD2 polymorphisms in a northern Han Chinese population, which may serve as a reference for elucidating the etiology of SCZ in different populations.

We also investigated the association between EFHD2 polymorphisms and SCZ to explore the pathogenesis of SCZ at the gene level, with the hope that SCZ may be diagnosed clinically at the gene level in the future. EFHD2 may also be a potential therapeutic target for the treatment of SCZ. SCZ is a complex disease that is regulated by multiple genes. The present results may contribute to future studies of the interactions between EFHD2 and other genes, and the interactions between multiple loci in the gene. Antipsychotic drugs are currently the main treatments for SCZ, but they can have serious side effects. Pharmacogenetic research has demonstrated that individual differences in responses to antipsychotic drugs are related to genetic variations. Thus, the early detection of polymorphic sites of target genes that may contribute to adverse reactions to antipsychotic drugs may contribute to individualized treatment. Future studies of the associations between EFHD2 and adverse reactions to antipsychotic drugs may therefore contribute to the development of new drugs and individualized treatment.

The present study has some limitations. First, the sample size was relatively small. Second, because the total length of EFHD2 is approximately 20 kb, the number of SNPs in this study may not be representative of the entire region. Thus, we cannot exclude the possible contribution of other SNPs in this gene to SCZ. Third, we did not perform functional analyses of the proteins that are expressed by genetic variations of EFHD2. Future studies that functionally characterize gene and protein expression profiles are therefore needed to validate our findings. Further studies that include larger cohorts of SCZ patients are also required to validate the role of EFHD2 in SCZ.

Conclusions

The present study demonstrated that the rs2473357 locus in EFHD2 may be related to SCZ in a northern Han Chinese population. We also revealed that sex is a crucial
risk factor for SCZ. The present findings may serve as reference data that will be useful for elucidating the etiology of SCZ.

Declaration of conflicting interest
The authors declare that there is no conflict of interest.

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