Gene 651 (2018) 100–105

Contents lists available at ScienceDirect

Gene

journal homepage: www.elsevier.com/locate/gene

Association between *SHANK3* polymorphisms and susceptibility to autism spectrum disorder

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ARTICLE INFO

Keywords:
Autism spectrum disorder
SHANK3
Single nucleotide polymorphisms
Neurodevelopmental disorders

ABSTRACT

Autism spectrum disorder (ASD), as one of neurodevelopmental disorders, affects about 1/160 of people worldwide. The etiology and pathogenesis of ASD remain elusive. Synapses are essential components of neurons and basic information transmission unit in the nervous system, adjusting behavior to environmental stimuli and controlling body functions, memories, and emotions. *SHANK3* is one of the synapse genes which play important roles in maintaining synaptic structure and function. *SHANK3* has been researched as a probably susceptibility gene for ASD. We investigated the association between polymorphisms in *SHANK3* and ASD in the Northeast Han Chinese population. A total of 470 subjects (229 cases and 241 controls) were enrolled in our case-control study. Five single nucleotide polymorphisms (SNPs) (rs756638, rs4824116, rs76268556, rs9616915, and rs75767639) in *SHANK3* were selected and genotyped. Our study did not identify a significant association of *SHANK3* SNPs with ASD in the Northeast Han Chinese population. Future studies need to test more *SHANK3* SNPs in large sample to demonstrate the association between SNPs in *SHANK3* and ASD.

1. Introduction

Autism spectrum disorder (ASD) is characterized by symptoms of neurodevelopmental disorders, including early-onset dysfunctions in communication, impairments in social interaction, and repetitive and stereotyped behaviors and interests (Lai et al., 2014). Patients develop the ASD-related symptoms when they are 12 to 18 months of age, and diagnosis is generally possible by the age of two years (WHO, 2016). WHO estimated that the global prevalence of ASD was 6.25‰ in 2013 (WHO, 2013). Moreover, the prevalence of ASD is increasing around the world during last decade (Sahin and Sur, 2015). ASD-affected males are as 4–5 times as females (Wingate et al., 2014). Nowadays, the patients with ASD usually have life-long disability, and specific medication for those patients is still deficient (Ratto and Mesibov, 2015).

ASD is a complex disease, and environmental and genetic factors influence development of this disease. Meta-analysis studies have identified possible environmental risk factors of ASD, including advanced reproductive age, complications during pregnancy, exposure to chemicals, and history of mental illness of mothers (Fang et al., 2015; Sandin et al., 2012; Von Ehrenstein et al., 2014; Wu et al., 2017). Those factors, however, can not explain development of ASD sufficiently. The heritability of ASD has been estimated to be 50% (Sandin et al., 2014). Accumulating twin- and family-based studies further indicate that genetic factors play critical roles in ASD, in that the concordance rate among monozygotic twins is higher (60–90%) than that rate among dizygotic twins (0–30%) (Ronald and Hoekstra, 2011; Sandin et al., 2014). Studies of linkage, association, and whole-genome or exome sequencing have strongly supported the association between genes and ASD (Murdoch and State, 2013; RK et al., 2017; Safari et al., 2017). Moreover, synaptic dysfunction is implicated in the pathogenesis of ASD (Lepeta et al., 2016; Zoghbi and Bear, 2012).

SHANK3 is synaptic multi-domain skeleton, maintaining synaptic transmission and plasticity (Monteiro and Feng, 2017). SHANK3 is highly expressed in developing neurons of cerebral cortex and

Abbreviations: ASD, Autism spectrum disorder; SNPs, single nucleotide polymorphisms; CHB, Han Chinese in Beijing population; MAF, minimum allele frequency; PCR, polymerase chain reaction; iMLDR, improved multiplex ligation detection reaction; HWE, Hardy-Weinberg equilibrium; LD, linkage disequilibrium; SH3, SRC homology 3; PDZ, postsynaptic density protein 95–discs large homologue 1–zonula occludens 1; PRO, a proline-rich region; SAM, asterile alpha motif; PSD, postsynaptic density

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https://doi.org/10.1016/j.gene.2018.01.078
Received 24 October 2017; Received in revised form 5 January 2018; Accepted 23 January 2018

cerebellum. *SHANK3* is located on chromosome 22q13.3. Mutations in *Shank3* in mice cause the substantial changes of behavioural phenotypes, including an increase in repetitive routines, altered social behavior, and anxiety-like phenotypes (Peca et al., 2011). These changes of behavioural phenotypes are seemingly similar to those described in patients with ASD. Thus, *SHANK3* is a probably susceptibility gene for ASD (Mashayekhi et al., 2016; Monteiro and Feng, 2017). However, the published results of the association between *SHANK3* polymorphisms and ASD are inconsistent in different ethnic populations (Mashayekhi et al., 2016; Shao et al., 2014). In this investigation, we designed a case-control study to investigate whether single nucleotide polymorphisms (SNPs) in *SHANK3* were associated with ASD in the Northeast Han Chinese population.

2. Materials and methods

2.1. Study subjects

A total of 470 subjects were included in our case-control study (229 cases and 241 healthy controls). All the cases (191 males and 38 females) were from the second department of Pediatrics, the first hospital in Jilin University and the Chunguang Rehabilitation hospital in Jilin Province. All the healthy controls (195 males and 46 females) were sex- and age-matched from the second department of Pediatrics, the first hospital in Jilin University without ASD or any other psychiatric disorders. The patients with ASD were diagnosed by Pediatric Neurology and Neurorehabilitation doctors using the Diagnostic and Statistical Manual of Mental Disorders (5th edition) (American Psychiatric Association., 2013). We excluded patients with fragile X syndrome, Rett syndrome, chromosomal abnormalities, and any neurological or psychiatric disorders. All the subjects were Northeastern Han Chinese. The study was approved by the ethics committee of Jilin University and the ethics committee of Chunguang Rehabilitation hospital. Written informed consent forms were obtained from parents or guardians of each subject.

2.2. Tag SNP selection

Tag SNPs for *SHANK3* were selected using the 1000 Genomes Browser database and Haploview 4.2 software. Selected options of Tag SNPs of *SHANK3* were Han Chinese in Beijing population (CHB), minimum allele frequency (MAF) cut-off of 10%, and r^2 cut-off of 0.8. Five tag SNPs (rs756638 G > A, rs4824116 C > T, rs76268556 C > T, rs9616915 T > C, and rs75767639 C > G) of *SHANK3* were finally selected. The positions of rs756638, rs4824116, rs76268556, rs9616915, and rs75767639 are located in 3′ Flanking, intron 12, intron 21, exon 6, and intron 12 of *SHANK3* respectively.

2.3. DNA extraction and SNP genotyping

Genomic DNA was extracted from peripheral blood samples using DNA extraction kits, according to the manufacturer's instructions (DP319 TIANamp Blood DNA Kit, TIANGEN BiotechCo. Ltd., Beijing, China).

Genotyping of each variant was performed using an improved multiplex ligation detection reaction (iMLDR) technique developed by Genesky Biotechnologies Inc. (Shanghai, China). Primers and probes for the multiplex polymerase chain reaction (PCR)-ligase detection reaction are listed in Table 1. PCR reaction mixture (20 μL) contained 1 × HotStarTaq buffer (Takara), 3.0 mM Mg^{2+}, 0.3 mM dNTP, 1 U HotStarTaq polymerase (Qiagen Inc), 1 μL of sample DNA, and 1 μL of each primer. PCR amplification was performed under the four steps: (1) 95 °C for 2 min; (2) 11 cycles at 94 °C for 20 s, 65 °C for 40 s, and 72 °C for 1.5 min; (3) 24 cycles at 94 °C for 20 s, 59 °C for 30 s, and 72 °C for 1.5 min; (4) a final extension at 72 °C for 2 min and hold at 4 °C. PCR products were purified by 5 U of shrimp alkaline phosphatase and 2 U of Exonuclease I at 37 °C for 1 h and at 75 °C for 15 min. The LDR mixture contained 1 μL of 10 × binding buffer, 0.25 μL of thermostable Taq DNA ligase, 0.4 μL of 1 μM 5′ ligation primers mixture, 0.4 μL of 2 μM 3′ ligation primers mixture, 2 μL of multiplex PCR product, and 6 μL of double distilled H_2O. LDR was performed under the two steps: (1) 38 cycles at 94 °C for 1 min and 56 °C for 4 min; (2) hold at 4 °C. A total of 0.5 μL of ligation product was denatured in 0.5 μL of the Liz-500 size standard and 9 μL Hi-Di formamide at 95 °C for 5 min, and run on the ABI3130XL sequencer. Raw data was analysed using GeneMapper Software 4.1 (AppliedBiosystems, USA). Each sample of 25 randomized subjects was genotyped again for quality control, and the repeat accuracy was 100%.

2.4. Statistical analyses

Goodness of fit Chi-squared test was used to determine whether the genotype distribution was in Hardy-Weinberg equilibrium (HWE). Chi-squared test and Fisher's exact test were used to check whether genotype and allele frequency distributions in cases were significantly different from those in controls. The online SNPStats analysis program was used to analyze the association between the five SNPs polymorphisms and ASD under five different genetic models (codominant, dominant, recessive, overdominant, and additive model) (Sole et al., 2006). The linkage disequilibrium (LD) degree between the loci was calculated using Haploview 4.2 software (Barrett et al., 2005). Haplotype analysis was performed using the SNPStats online analysis program. Statistical analyses were performed using SPSS21.0 software unless otherwise specified. The statistical tests were 2-tailed, and statistical significance was defined as $P < 0.05$.

3. Results

The mean age of the cases and controls was four years. No significant sex or age differences were found between patients with ASD and healthy controls ($P > 0.05$). The genotype distributions of the five *SHANK3* SNPs in control group were in HWE (all $P > 0.05$). Genotyping rates of rs756638 and rs75767639 were 99.4% and 99.8%, and those of the other three sites were 100%. The mutant homozygote frequencies of rs756638, rs4824116, rs76268556, rs9616915, and rs75767639 in cases were 5.7%, 0.4%, 0.0%, 1.3%, and 0.4% respectively, and those frequencies in controls were 2.9%, 1.2%, 1.2%, 1.2%, and 1.2% respectively. The mutant allelic frequencies of rs756638, rs4824116, rs76268556, rs9616915, and rs75767639 in cases were 19.2%, 9.4%, 9.0%, 10.3%, and 8.8% respectively, and those frequencies in controls were 16.7%, 8.9%, 7.5%, 9.5%, and 8.9% respectively. The results of Chi-squared test and Fisher's exact test showed that the genotypic and allelic frequencies of the five *SHANK3* SNPs in patients with ASD were not different from those in controls ($P > 0.05$) (Table 2).

We further investigated the association of *SHANK3* polymorphisms and ASD under codominant, dominant, recessive, overdominant, and additive model. Owing to no mutant homozygote (TT) observed in cases for rs76268556, the results of codominant and recessive models were not calculated. Our results showed that there was no significant association between the five SNPs and ASD under any inheritance model (Table 3).

The analysis of LD showed that the five SNPs in *SHANK3* were not in LD (Fig. 1). Haplotype analysis did not identify risk haplotypes, and the frequency of any haplotype was not different between patients with ASD and controls ($P > 0.05$) (Table 4).

4. Discussion

In the present study, we found that no association between the polymorphisms of rs756638, rs4824116, rs76268556, rs9616915, and rs75767639 and risk of ASD in Northern Chinese Han. Moreover, there