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Polymorphisms of *Shank3* gene in Chinese Han Children with Autism Spectrum Disorders (ASD): A case-control study

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Abstract

This study investigated the co-relationship between single nucleotide polymorphisms (SNPs) variations of *SHANK3* (SH3 and multiple ankyrin repeat domains 3) gene and childhood autism in Chinese Han populations. high-resolution method (HRM) were used to examine 11 polymorphisms of *SHANK3* in 100 patients with ASD compared to 100 health controls. Risk estimates were expressed as odds ratio (OR) and 95% confidence interval (CI). Overall there were significant differences in the allele distributions of rs9616816, rs6010061 and rs756638 (for rs9616816, OR=1.918, 95%CI=1.287-2.858, P=0.001; for rs6010061, OR=0.517, 95%CI=0.291-0.919, P=0.023; for rs756638, OR=0.506, 95%CI=0.296-0.866, P=0.012; respectively). Logistic regression analyses confirmed that these three SNPs were directly linked with significant risk of ASD. Our data indicate that *SHANK3* gene polymorphisms may play a vital role in ASD genetic susceptibility in Chinese Han populations.

Introduction

The autism spectrum disorders (ASD), first reported in the early 1940sby psychiatristsis a severe neuropsychiatric disorder syndrome [1,2]. ASD is characterized by impairments of sociality and communication, lacking of reciprocal social interaction or responsiveness and repetitive or stereotyped behaviors (citation), the Onset of ASD mainly occurs in early childhood [3]. Despite the etiology of autism are largely unknown, it is widely accepted that autism is a multifactorial disease with a complex interaction of genetic and environmental factors [1,5,6]. In the past decades, though promising progress has been reported, ASD is still an urgent challenge for the public health [7].

Research proved that cause of ASD may reside in abnormalities at the synapse [8,9]. The *SHANK3* gene, encodes a synaptic scaffolding protein [10]. In human beings, *SHANK3* is expressed preferentially in cerebral cortex and cerebellum [11,12]. With its multiple protein interaction domains, this molecule directly or indirectly connects with neurotransmitter receptors and cytoskeleton proteins [13,14]. It also participates in the formation, maturation and enlargement of dendritic spines and is essential for the formation of functional synapse [10,15,16]. More importantly, a number of studies have been performed on *SHANK3* polymorphisms with autism risk in different populations. However, the results were still controversial. Only one study of polymorphisms in *SHANK3* found significant difference between ASD cases and controls [17], while the others were negative [18,19,20].

The genetic variations of *SHANK3* of ASD in Chinese Han population has been reported by Qin and Shao, however, it's not sufficient to demonstrate the co-relation between *SHANK3* gene with other SNPs for the lack of diversity of the investigated SNPs. Therefore, further research about the co-relation between genetic variations of *SHANK3* with ASD in Chinese Han populations is necessary and important. So far 21586 SNPs (according to NCBI) of *SHANK3* gene has been reported. Accordingly, we selected 11 tag SNPs of *SHANK3* gene in 100 cases and 100 controls to ascertain the association between this SNP and ASD susceptibility in Chinese Han children to gain a better understanding of the way it exerts its effect on ASD.

Material and method

Participants

A total of 100 ASD patients (76 males and 24 females, 2-12 years) and 100 healthy controls (69 males and 31 females, 2-12 years) were enrolled from Zhongnan Hospital of Wuhan

University, Hubei Provincial Maternal and Child Health Hospital. All patients met DSM-IV diagnostic criteria for ASD. Written informed consent was obtained from each participant, and the study protocol was approved by the ethics committees of Zhongnan Hospital of Wuhan University.

Genotyping

Genomic DNA was extracted from the blood using a TIANamp Blood DNA Kit (TIANGEN, BeiJing, China). Eleven SNPs were selected from the HapMap HCB database with the criteria used in our SNP selection procedure [a minor allele frequency over 0.1 and tag SNPs with an r^2 value above 0.8] to examine the association between *SHANK3* and ASD (Table 1). SNPs were genotyped by high-resolution melting of small amplicons on LightScanner 96 instrument (Idaho Technology, USA). Primer details and product lengths are shown in table S1. About 5% of the samples were randomly selected using direct PCR sequencing (Life Technologies Corporation, Shanghai, China) and the concordance was 100%.

Table 1: Characteristics of SNPs in shank3 gene cluster

SNP	Position[1]	Minor allele	Major allele	MAF[2]
rs2301584	51171497	A	G	0.223
rs2341011	51139635	T	C	0.321
rs41281537	51171667	A	G	0.058
rs5770820	51150473	A	G	0.253
rs5770992	51146139	G	A	0.108
rs6010061	51151724	C	T	0.417
rs6010065	51158017	C	G	0.490
rs756638	51171693	A	G	0.299
rs8137951	51165664	A	G	0.374
rs9616816	51123505	A	G	0.362
rs9616915	51117580	C	T	0.358

[1] Position in basepairs was derived from dbSNP Build 137. Based on NCBI Human Genome Build 37.3 (November, 2014) of chromosome 22
[2] MAF, minor allele frequency

Table S1. Amplification primers utilized in the genotype

SNP		Primers (5'→3')	product length (bp)	Tm (℃)
rs9616816	Forward Reverse	GCTCTCAGCATGGAAAGA TCCCATCACTGTTGTTTT	57	54.2
rs6010061	Forward Reverse	GGAGTTTTCTCTCCATTCATATCTT CTTAAGCACCATACTCC	60	55
rs756638	Forward Reverse	TGTGTCTGTCCCTCATACC CATGTGGTCCAGGCTGA	102	54.5
rs6010065	Forward Reverse	TGGTACTTCTGCGTCGG GCCAGTACAGGGCTCC	89	59
rs2301584	Forward Reverse	GTTCCGCTTCACCTCCTT GCCTCAGGACTGGAGCA	69	57
rs41281537	Forward Reverse	GCTCAGTTGCCTGCTTG CCGGTATGAGGGACAGA	86	58
rs2341011	Forward Reverse	TCCGCTTCACCTCCTTT GCCTCAGGACTGGAGCA	67	56.8
rs5770992	Forward Reverse	TGGTCAGAATTTTCAC TTATCTACATGGGGTT	50	45
rs5770820	Forward Reverse	CTCTAGGGAGCAGGGAGAC GACCAGCAGAAAGAAGCAA	112	55
rs9616915	Forward Reverse	TCTCCACGACCACGC CTCCTGCCAGCCATT	52	63
rs8137951	Forward Reverse	ATGTCATACATACTATTTTTGCATT TAGCACAAAGCCAGGAA	55	53.6

Statistical methods

Hardy-Weinberg Equilibrium and allele frequency distributions were analyzed by the chi-square test (SPSS, version 18.0). Each genotype of examined polymorphisms was assessed by logistic regression analyses under the additive (major homozygotes versus heterozygotes versus minor homozygotes), dominant (major homozygotes versus heterozygotes plus minor homozygotes) and recessive (major homozygotes plus heterozygotes versus minor homozygotes) models of inheritance after adjusting for sex and age, respectively (SPSS, version 18.0). Linkage disequilibrium (LD) analysis of SNPs and the haplotype association were analyzed using Haploview 4.2 and SHEsis software. P values less than 0.05 were considered statistically significant.

Results

Genotype and Allele Frequencies

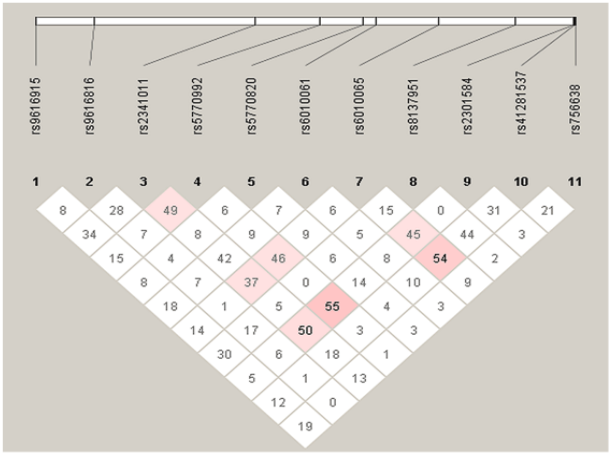
No significant deviation from HWE was observed for all tested SNPs in the control groups (P > 0.05). As shown in table 2, logistic regression analysis revealed that rs9616816 was associated with ASD in both additive model [OR = 1.791, 95% CI (1.217-2.635), p = 0.003] and recessive model [OR = 2.569, 95% CI (1.382-4.774), p = 0.003], rs6010061 and rs756638 were also associated with ASD in both additive model [OR = 0.550, 95% CI(0.320-0.946), p = 0.031; OR = 0.510, 95% CI(0.296-0.878), p = 0.015] and dominant model [OR = 0.472, 95% CI (0.242-0.922), p = 0.028; OR = 0.461, 95% CI (0.248- 0.858), p = 0.014].

Table 2: Risk estimate based on the distributions of genotype and allele frequency.

SNP	genotype	Control (n = 100)	Case (n =100)	Allele OR(95% CI), P value[1]	Additive OR(95% CI), P value[2]	Dominant OR(95% CI), P value[2]	Recessive OR(95% CI), P value[2]
Rs9616816	GG	31	19				
	GA	45	37				
	AA	24	44	1.918 (1.287-2.858), **0.001**	1.791 (1.217-2.635), 0.003	1.927(0.994-3.737), 0.052	2.569(1.382-4.774), 0.003
rs6010061	TT	69	82				
	CT	25	15				
	CC	6	3	0.517 (0.291-0.919),**0.023**	0.550 (0.320-0.946), 0.031	0.472 (0.242-0.922), 0.028	0.436 (0.104-1.818), 0.254
rs756638	GG	61	77				
	GA	34	21				
	AA	5	2	0.506 (0.296-0.866),**0.012**	0.510 (0.296-0.878), 0.015	0.461 (0.248-0.858), 0.014	0.386 (0.072-2.064), 0.266
rs6010065	GG	28	37				
	GC	43	39				
	CC	29	24	0.755 (0.509-1.119), 0.161	0.788 (0.543-1.142), 0.208	0.674 (0.368-1.233), 0.200	0.764 (0.403-1.452), 0.412
rs2301584	GG	70	71				
	GA	26	27				
	AA	4	2	0.896 (0.526-1.524), 0.684	0.920 (0.542-1.562), 0.759	0.992 (0.536-1.834), 0.979	0.468 (0.083-2.637), 0.389
rs41281537	GG	79	73				
	GA	18	26				
	AA	3	1	1.194 (0.666-2.141), 0.552	1.157 (0.647-2.068), 0.623	1.355 (0.703-2.612), 0.365	0.304 (0.031-3.002), 0.308
rs2341011	CC	49	40				
	CT	41	47				
	TT	10	13	1.310 (0.864-1.987), 0.204	1.309 (0.861-1.988), 0.208	1.457 (0.829-2.560), 0.191	1.325 (0.548-3.205), 0.532
rs5770992	AA	60	65				
	AG	32	28				

	GG	8	7	1.189 (0.769-1.838), 0.437	0.859 (0.552-1.338), 0.502	0.805 (0.453-1.433), 0.462	0.871 (0.302-2.512), 0.799
rs5770820	GG	23	33				
	GA	44	34				
	AA	33	33	0.818 (0.552-1.212), 0.317	0.829 (0.578-1.188), 0.307	0.568 (0.301-1.073), 0.082	0.991 (0.548-1.791), 0.975
rs9616915	TT	87	87				
	CT	12	11				
	CC	1	2	1.077 (0.506-2.295), 0.847	1.027 (0.501-2.104), 0.942	0.940 (0.407-2.169), 0.885	2.174 (0.187-25.238), 0.535
rs8137951	GG	55	58				
	GA	36	32				
	AA	9	10	0.950 (0.609-1.481), 0.821	0.955 (0.627-1.453), 0.828	0.886 (0.505-1.554), 0.672	1.116 (0.430-2.895), 0.822

The minor C allele of rs6010061 was associated with a lower risk of ASD [OR = 0.517, 95% CI (0.291-0.919), p = 0.023] and the minor A allele of rs756638 was associated with a lower risk of ASD [OR = 0.506, 95% CI (0.296-0.866), p = 0.012], while carriers of the rs9616816 A allele were associated with a higher risk of ASD [OR = 1.918, 95% CI (1.287-2.858), p = 0.001]. Other SNPs, like rs6010065, rs2301584, rs41281537, rs2341011, rs5770992, rs5770820, rs9616915, and rs8137951, did not show any association with ASD.



Figure

Linkage disequilibrium and haplotype analysis

The Haploview 4.2 software was used for linkage disequilibrium analysis, and none of SNPs of this section are closely linked (D' and r2 > 0.85). The LD block of 11 SNPs were constructed (Figure 1). Table 3 presents the haplotype frequencies (≥ 3%) of three positive polymorphisms in patients and controls. The most common haplotype A-T-G, G-C-G, G-T-A was assigned as the reference group in risk estimates. But the haplotype ATG may increase the risk of autism (OR: 2.702, 95%:1.769~4.126, p < 0.01). Details are listed in table S1.

Table 3: Haplotype analysis in the control and the autism group

Haplotypes	Cases (Freq.)	Controls (Freq.)	χ^2	P	Odds ratio (95%CI)
A C A	2.90(0.015)	2.70(0.013))	/	/	/
A C G*	12.17(0.061)	16.97(0.085)	0.875	0.35	0.695 [0.324~1.494]
A T A*	12.52(0.063)	20.88(0.104)	2.326	0.13	0.570 [0.274~1.183]
A T G*	97.41(0.487)	52.45(0.262)	21.676	3.3E-06	2.702 [1.769~4.126]
G C A	2.47(0.012)	3.43(0.017)	/	/	/
G C G*	3.45(0.017)	13.90(0.070)	6.626	0.01	0.234 [0.071~0.773]
G T A*	7.11(0.036)	17.00(0.085	4.365	0.04	0.395 [0.161~0.970]
G T G*	61.96(0.310)	72.67(0.363)	1.367	0.24	0.779 [0.512~1.184]

Discussions

In this case-control study, we investigated the relationship between the 11 tag SNPs in *SHANK3* gene and the risk of ASD in the Chinese population. Among the 11 SNPs, three SNPs (rs9616816, rs756638, rs6010061) were found to be significantly associated with the risk of ASD in Chinese Han populations.

Since the first report of *SHANK3* mutations in ASD was published by Moessner et al. in 2007, several studies have been investigated

the relationship between *SHANK3* gene polymorphisms and ASD in different populations. Qin found none of the five SNPs was significant evidence ($P < 0.05$) for preferential transmission of an allele by FBAT in all samples [18]; Sykes's data suggested that *SHANK3* deletions may be limited to lower functioning individuals with autism[19]; Chien's research revealed that the 5 tag SNPs (rs2341011, rs5770992, rs5770820, rs6010065,and rs2301584) were not significant statistically [19]. However, there were few positive results of association between *SHANK3* polymorphisms and ASD. Shao's study of rs9616915 polymorphisms in *SHANK3* found significant difference between ASD cases and controls in Chinese Han population [17], while the others reported that *SHANK3* might not represent a major susceptibility gene for ASD. The inconsistency with these pioneer works is possibly due to the difference of the sample size, individual genetic background, research design and environmental factors. However, the results were not fully consistent with previous reports. In the present study, we found the rs9616816, rs756638 and rs6010061 polymorphisms in the *SHANK3* gene has a statistically significant association with ASD susceptibility and may affect the subject susceptibility toward autism in the Chinese Han population. We established genotyping methods of 11 SNPs in the *SHANK3* gene cluster by high-resolution melting and successfully found both the rs9616816 and rs6010061 were associated with ASD risk. The protective role of rs9616816 A allele against the risk of ASD suggested *SHANK3* a possible candidate gene involved in the pathogenesis of ASD. Furthermore, Analysed with three models for genotype distributions, the association between *SHANK3* SNPs (rs9616816, rs756638, rs6010061) and ASD remained significant after performing statistical adjustments for age and sex. This result supported previous reports that *SHANK3* gene is a susceptible predictor of ASD risk factors [21,22,23,24,25].

Till now, little information is known about the role of *SHANK3* gene in the diverse pathological processes to ASD children. *SHANK3* gene, encodes a protein of the postsynaptic density of excitatory synapses, had been shown to bind to neuroligin, which, form a complex at glutamatergic synapses. In humans, *SHANK3* was found expressed predominantly in cerebral cortex and cerebellum [4,12, 26]. Durand and coworkers then identified two alterations in *SHANK3* in subjects with an ASD, one is a de novo insertion of a G nucleotide in exon 21 of SHANK3, which leads to a frame-shift and presumed loss of function; the other was found in an unrelated family with a de novo deletion of terminal 22q13, with the breakpoint in intron 8 of *SHANK3* [27]. Genetic and functional data implicate *SHANK3* as a potential genic cause of ASD, which lead us to seek to further assess the involved polymorphisms and associated phenotypic outcomes. Recent studies indicate that autism is a disease of polygenic inheritance. Analysis of polymorphisms in the *SHANK3* gene allows to effectively screen for autism risk [17]. Most previously reported studies narrowed on the mutations region. In our study, we covered the whole region of the *SHANK3* gene, 11 tag SNPs, spreading in coding regions, 5′- and 3′- UTR regions, were selected and studied in our cohort. Positive SNPs, found only in intron (rs9616816 and rs6010061) and 3′ untranslated region (3′ UTR) (rs756638) of *SHANK3* gene, indicated potential mechanism on the affection of the expression of *SHANK3* by binding with transcription factors or micro RNA, could be altering the interaction of Shank with miRNAs.

The correlation between genotype and phenotype is very complicated, both genetic and environmental infaectors have significant effect on ASD. The further investigation is that positive SNPs how to regulate the expression of *Shank3* gene by micRNA to reveal the role *shank3* gene on the pathogenesis of ASD.

Conclusions

Our study supports that *SHANK3* be a critical gene for the etiology of autism in Han Chinese population and three SNPs of *SHANK3* gene potentially function as risky factor for ASD upon further validation and functional studies.

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