Original Article
The single nucleotide polymorphism study on the SHANK3 and NLGN3 gene in association with autism in Wenzhou children

Yongzhong Zhao[1], Chuang Yang[1], Haixi Lin[1], Yan Liang[1], Weiqian Wang[1], Hong Chen[1], Meihao Wang[1], Huiru Liu[1], Senjian Luo[2], Yanhong Yi[1]

[1]Department of Psychiatry, The First Affiliated Hospital of Wenzhou Medical University, Wenzhou Medical University, Wenzhou 325000, Zhejiang Province, China; [2]Department of Psychiatry, Yueqing Third People's Hospital, Yueqing 325604, Zhejiang Province, China

Abstract: Objective: To study the SHANK3 and NLGN3 Gene in Association with Autism in Wenzhou Children for its Single nucleotide polymorphism; Methods: It studied a Wenzhou sample of origin consisting of 100 unrelated patients with ASDs. 31 tagging SNPS within SHANK3 and NLGN3 gene with a minor allele frequency (MAF) greater than 5% in the population were selected to capture the majority of the common variations. Matrix-assisted laser desorption/ionization time-of-flight mass spectrometry was used for SNP genotyping; Results: A significant genetic association between an intronic SNP rs9616915 and female ASDS was observed, with T-allele having an increased risk to develop to autism. Significant differences of allele frequencies were detected for 3SNPs in NLGN3 gene contrasted between cases and controls for male and female samples separately (male: rs11795613, rs4844285 and rs5981079, female: rs11795613, rs4844285 and rs7051529). Neither single SNP nor Haplotype in SHANK3 and NLGN3 was detected to show significant association with ASDS, even after gender stratification analysis. Conclusion: The SNPS with rs9616915 and rs13057681 of SHANK3 gene, the SNPS with rs11795613, rs4844285 and rs5981079 of NLGN3 gene were associated with Autism in Wenzhou Children.

Keywords: The single nucleotide polymorphism study on the SHANK3 and NLGN3 gene in association with autism in Wenzhou children

Introduction

Autism is a heritable developmental disorder involving macroscopic early brain overgrowth in the majority of cases and dysfunction that affects several cortical and subcortical regions mediating autistic symptoms, including prefrontal and temporal cortexes [1-4]. The underlying cortical defects remain uncertain. Despite the early diagnosable onset, in more than 40 studies, the average age of patients with autism in postmortem analyses were 22 years [5].

The heritability of autism is the proportion of autism that can be explained by genetic variation; if the heritability of a condition is high, then the condition is considered to be primarily genetic. Autism has a strong genetic basis, although the genetics of autism is complex and

it is unclear whether autism (ASD) is explained more by multigene interactions or by rare mutations with major effects [6, 7].

Genetic linkage analysis has been inconclusive; many association analyses have had inadequate power. For each autistic individual, mutations in more than one gene may be implicated. Mutations in different sets of genes may be involved in different autistic individuals. There may be significant interactions among mutations in several genes, or between the environment and mutated genes [8, 9]. By identifying genetic markers inherited with autism in family studies, numerous candidate genes have been located, most of which encode proteins involved in neural development and function [10-12]. However, for most of the candidate genes, the actual mutations that increase the risk for

autism have not been identified. Typically, autism cannot be traced to a Mendelian (single-gene) mutation or to single chromosome abnormalities such as fragile X syndrome or 22q13 deletion syndrome. Although the fraction of autism traceable to a genetic cause may grow to 30-40% as the resolution of array CGH improves, several results in this area have been described incautiously, possibly misleading the public into thinking that a large proportion of autism is caused by CNVs and is detectable via array CGH, or that detecting CNVs is tantamount to a genetic diagnosis [13, 14]. The Autism Genome Project database contains genetic linkage and CNV data that connect autism to genetic loci and suggest that every human chromosome may be involved. It may be that using autism-related subphenotypes instead of the diagnosis of autism per se may be more useful in identifying susceptible loci.

SH3 and multiple ankyrin repeat domains 3 (Shank3), also known as proline-rich synapse-associated protein 2 (ProSAP2), is a protein that in humans is encoded by the SHANK3 gene on chromosome 22. Additional isoforms have been described for this gene but they have not yet been experimentally verified [15, 16].

Mutations in this gene are associated with autism spectrum disorder. This gene is often missing in patients with 22q13.3 deletion syndrome, although not in all cases.

This gene is a member of the Shank gene family. Shank proteins are multidomain scaffold proteins of the postsynaptic density that connect neurotransmitter receptors, ion channels, and other membrane proteins to the actin cytoskeleton and G-protein-coupled signaling pathways. Shank proteins also play a role in synapse formation and dendritic spine maturation.

Neuroligin is a cell surface protein (homologous to acetylcholinesterase and other esterases) that binds to synaptic membranes. Neuroligins organize postsynaptic membranes that function to transmit nerve cell messages (excitatory) and stop those transmissions (inhibitory) [15-17]; by this way, neuroligins help to ensure signal transitions between nerve cells. Neuroligins also regulate the maturation of synapses and ensure there are sufficient receptor proteins on the synaptic membrane.

Wenzhounese is a prefecture-level city in south-eastern Zhejiang province in the People's Republic of China. Wenzhou is located at the extreme south east of Zhejiang Province.

Wenzhou was a prosperous foreign treaty port, which remains well-preserved today. It is situated in a mountainous region and, as a result, has been isolated for most of its history from the rest of the country, making the local culture and language very distinct not only from the rest of China but from neighboring areas as well. It is also known for its emigrants who leave their native land for Europe and the United States, with a reputation for being entrepreneurs who start restaurants, retail and wholesale businesses in their adopted countries. People of Wenzhou origin make up a large number of ethnic Chinese residents of Italy (where they made the 90% of all Chinese residents), France, and Spain. So it has a special significance to research SNP of Gene associated with Autism in China.

Materials and methods

Subject

It studied a Wenzhou sample of origin consisting of 100 unrelated patients with ASDs and 100 ethnically and geographically matched controls in this study. All affected subjects were diagnosed according to the diagnostic and statistical manual of mental disorders criteria or international classification of diseases-10 from outpatients of children's hospital of Wenzhou Medical university. All controls were randomly drawn from outpatients of our hospital with no personal history of psychiatric disorders.

All 100 patients were from the First Affiliated Hospital of Wenzhou Medical University, from January 2013 to January 2014. They were divided in two groups randomly, 77 boys and 73 girls with average age of 3.26 0.3 years, were constitute the control group; 76 boys and 74 girls with average age of 3.11 0.9 years, were constitute the treatment group. There was no difference for Gender, Age and hospitalization time in the two groups (P>0.05).

All the subjects were excluded: any hepatitis or HIV infection, other causes of liver damage, autoimmune disorders and neoplasm. In the research process, the cases were untreated

with the intervention by antivirals, immunosuppressant or immunomodulators, at least 6 months before collecting serum.

Medical Ethics Committee of Wenzhou Medical university approval was obtained and all involved patients had previously provided their written, informed consent to have their clinical and pathogenic information used for research.

Extracted DNA

Blood samples were collected in the first time visited hospital. Serum was separated, promptly, which was Stored under -80°C. DNA pure Mini Kit (Cat. No. CW0624) were purchased from the CW Biotech. Ltd. Accordance with the instructions, it extracted nucleic acids: DNA in the serum separately.

PCR and MAF

DNA kit ((Invitrogen, CA, USA) was used to extract total DNA from 500 μl blood. The DNA was eluted into 50 μl of H_2O.

After that, the target DNA genes were determined by PCR using the PE 7000 Sequence Detection System (ABI PRISM, USA) with β-actin as an internal standard. The PCR mixtures were treated for 10 min at 42°C, then 2 min at 93°C and were followed by 40 cycles of amplification: denaturation at 93°C for 45 sec, renaturation at 55°C for 45 sec. The fluorescence signal was collected at the renaturation step.

SNPS selection and genotyping: based on the genotype data from the SNP database and international hapmap project, 31 tagging SNPS within SHANK3 and NLGN3 gene with a minor allele frequency (MAF) greater than 5% in the population were selected to capture the majority of the common variations. Matrix-assisted laser desorption/ionization time-of-flight mass spectrometry was used for SNP genotyping.

Statistical analysis of data

Statistic analysis: the hardy-Weinberg equilibrium (HWE) was assessed with chi-square test. SNPS were used in case-control study for allelic, genotypic and haplotypic association and were also performed with genders stratification.

The data are changed into normal distribution with logarithm if the original data are positive skewness distribution. If the Data are homogenous, Analysis of variance, Student-Newman-Keulsa and Pearson's correlation will be used. If the data are not homogenous, Kruskal-Wallis, Games-Howell test, as well as spearman's correlation analysis will be used. All the analyses were carried out using the SPSS17.0 software (SPSS Inc, Chicago, IL, USA). Values less than 0.05 were considered to be statistically significant.

Results

Association analysis of SNPS of SHANK3 with autism spectrum disorders

A significant genetic association between an intronic SNP rs9616915 and female ASDS was observed, with T-allele having an increased risk to develop to autism (P=0.038, OR=1.955, 95% CI: 1.035-3.69), and the log-additive model was accepted as the best inheritance model fitting this data (P=0.023, OR=2.28, 95%: 1.09-4.74). Haplotype-specific association analysis revealed that the haplotype T-T (rs9616915-rs13057681) showed significant association with female ASDS and has increased risk to develop to ASDS (P=0.039, OR=1.925, 95% CI: 1.029-3.604).

Association analysis of SNPS of SHANK3 and NLGN3 with autisms spectrum disorders

Neither single SNP nor Haplotype in SHANK3 and NLGN3 was detected to show significant association with ASDS, even after gender stratification analysis.

Association analysis of SNPs of NLGN3 with autism spectrum disorders

Significant differences of allele frequencies were detected for 3SNPs in NLGN3 gene contrasted between cases and controls for male and female samples separately (male: rs11795613, rs4844285 and rs5981079, female: rs11795613, rs4844285 and rs7051529). In addition, significant differences in genotype distributions of these three SNPs were also detected in female samples under a log additive model: rs11795613, P=0.036; rs4844285, P=0.036; rs7051529, P=0.038.

Haplotype-specific association analysis revealed that 4-SNPs haplotype A-G-T-T, 3-SNPs

Table 1. Association analysis of SNPs of NLGN3 with autism spectrum disorders

SNPs	P	OR	95% CI
Serial-a			
A-G-T-T	0.031	1.633	1.046-2.549
A-G-T	0.015	1.758	1.112-2.778
A-G	0.0113	1.803	1.14-2.854
T-T	0.047	1.571	1.005-2.345
Serial-b			
G-A	0.0184	0.575	0.361-0.913
Serial-c			
A-G-T	0.03	2.16	1.071-4.357
A-G	0.035	2.147	1.048-4.400
Serial-d			
G-A	0.035	0.466	0.227-0.954
G-T	0.044	0.383	0.147-0.999

haplotype A-G-T, 2SNPs haplotype A-G and T-T play a role as a susceptibility factor for male ASDs in **Table 1** (Serial-a). 2SNPS haplotype G-A play a role as a protective factor for male ASDs in **Table 1** (Serial-b). Moreover, the haplotype A-G-T and A-G play a role as a susceptibility factor for female ASDs in **Table 1** (Serial-c), the haplotye G-A and G-T play a role as a protective factor for female ASDs in **Table 1** (Serial-d).

Discussion

Wenzhou Medical College is a well-known college specializing in ophthalmology (national level key discipline), as well as the provision of other medical courses. Several of Wenzhou's major hospitals are affiliated to this college. In 2013, the Chinese Ministry of Higher Education china upgraded WMC's status to that of a medical university; it has thus been renamed the Wenzhou Medical University.

The Ministry of Education of the People's Republic of China approved the establishment of Wenzhou-Kean University on November 16, 2011. It is one of the first two Chinese-American cooperatively run universities with legal person status, the other one being NYU Shanghai inaugurated on Oct. 15, 2012.

Few scientists think that the leaders of the tech world actually have an autism spectrum disor-

der (ASD), which can range from the profound social, language and behavioural problems that are characteristic of autistic disorder, to the milder Asperger's syndrome. But according to an idea that is creeping into the popular psyche, they and many others in professions such as science and engineering may display some of the characteristics of autism, and have an increased risk of having children with the full-blown disorder [17-19].

Back in 1997, for example, he concluded that fathers of children with autism were more than twice as likely to be engineers as were fathers of non-autistic children8. But autism researchers Christopher Jarrold and David Routh at the University of Bristol, UK, pointed out that Baron-Cohen reported the analysis of data only for engineers, not for the other occupations surveyed. After analyzing the same data9, they found that fathers of children with autism were more likely to work in medicine, science and accountancy, as well as engineering, and less likely to have manual occupations. They suggested that these fathers were simply more likely to have reached a higher level of education [20, 21].

Ph.D Rich Stoner hypothesized that, In 2014, such a disturbance is present in the neocortex of children with autism and that it is detectable in the prefrontal and temporal cortexes, as reported in previous studies of children with autism that used magnetic resonance imaging (MRI), functional MRI, gene expression, and neuron count.

Indeed, researchers say that several other factors could explain the seeming correlation between autism and science or engineering. A 2010 analysis of autism diagnoses in California 11 did not find that autism clustered preferentially around areas rich in IT industry. Instead, it found that clusters tended to occur in areas where parents were older and educated to a higher level than were parents in surrounding areas. "Virtually all of these clusters were also clusters of higher education", says lead author Irva Hertz-Picciotto, an epidemiologist at the University of California, Davis [22-24].

Autism spectrum disorders (ASDS) are severe neurodevelopmental syndrome with a complex genetic etiology, which characterized by impaired reciprocal social interactions, deficient

communication, restricted interests and stereotyped activity patterns.

ASDS are achnowledged to be among the most heritable neuropsychiatric disorders and etiologically heterogeneous, probably associated with a combination of the effects of multiple genes and environemental facors, and the role of genetic factors in the pathogenesis of ASDS has been definite established. Many independent genome-wide scans for ASDS susceptibility loci have been carried out. The region on chromosome 2q, 7q, 22q, Xq and Xp stands out as the region of suggetive linkage to atiology of ASDS in many independtent genome-wide scans. The synaptic hypothesis was also proposed that alteration of synaptic homeostasis and impairments in synapse development is thought to be a major cause of brain disorders such as autism and mental retardation [25, 26]. A gentics topic in ASDS has emerged focusing on identification of the synaptic genes contributing to the formation and function of the synapse. Of particular interests are synaptic genes of the NLGN3-SHANK3 pathway in ASDS pathogenesis. Two synaptic associated gene NLGN3 and SHANK3 selected in this study, are located within ghese regions. By using the case-control association analysis with many tagging SNPS of these two syanptic gnes, we will investigate the relationship between ASDS and the sigle SNP and the haplotypes, also analysis with gender stratification [27, 28].

It has also heen considered that genes contributing to ASDS are likely a combination of rare variants in fewer individuals with a dramatic effect and common variants in majority invividuals with small increments of risk [29].

In Prospective study, we studied the rare mutations of synaptic gene NLCN3, SHANK3 with autism spectrum disorders in our hospital. It found that a specific mutation of SHANK3 gene (c.203T>C) is confirmed by a molecular screening in another ethinic population, suggested this missense mutation may be a cause of ASDS. However, functional studies will be required to confirm that this mutation is indeed pathogenic.

Disclosure of conflict of interest

None.

Address correspondence to: Yang Chuang, Department of Psychiatry, The First Affiliated Hospital of Wenzhou Medical University, Wenzhou Medical University, Fuxue Lane 2#, Wenzhou 325000, Zhejiang Province, China. Tel: 0577-55578166; Fax: 0577-555780339; E-mail: chuangge87@126.com

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