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Association of Granzyme B Gene Polymorphism with Autism Spectrum Disorder in Northeast Han Chinese Population

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Abstract

Background: Autism Spectrum Disorder (ASD) is a common neurodevelopmental disorder. Several susceptibility genes were found in the genome-wide study of ASD. There are few reports of the association between granzyme B (GZMB) and ASD.
Objectives: This study aimed to investigate the association between ASD and GZMB polymorphism in the northeast Han Chinese population.
Methods: A case-control study was conducted in Changchun city, a northeast city of China, from June 2014 to November 2014. We enrolled 268 Han population Chinese children in a case-control study, including 85 ASD patients and 183 healthy controls. We also recruited 67 nuclear family trios. Three single-nucleotide polymorphisms (SNPs) (rs2236338, rs10873219, and rs8192917) were selected and genotyped. The association between the GZMB SNPs and ASD was analyzed using the Transmission Disequilibrium Test (TDT).
Results: No statistical differences were found in allele and genotype frequency of the three SNPs of the GZMB gene between the case and control groups (all $P > 0.05$). Our study showed that rs2236338-rs10873219-rs8192917 G-T-G ($P = 0.41$) and G-G-G ($P = 0.59$) haplotypes were not associated with ASD. Moreover, TDT showed negative results ($P = 0.885$ for rs2236338, $P = 0.900$ for rs10873219, and $P = 0.900$ for rs8192917).
Conclusions: There was no association between the three SNPs in GZMB and ASD. Further studies need to determine and verify the relationship between the GZMB gene and ASD.

Keywords: Alleles, Autism Spectrum Disorder, GZMB Protein, Genome-Wide Association Study, Genotype, Granzymes, Human, Polymorphism, Single Nucleotide

1. Background

Autism Spectrum Disorder (ASD) is a common neurodevelopmental disorder with three abnormal characteristics, including deficit in social interaction, impairment in communication skills, and restricted repetitive behaviors (1). Previous studies reported that the prevalence of ASD is about 1% (2), with a male to female ratio of 4:1 (3). Obviously, genetic factors play a pivotal role in the etiology of ASD. Twin studies showed that the estimated heritability of ASD is close to 90% (4). The risk of ASD is greater among siblings than in the general population (5). In recent decades, an intense interest has focused on ASD with an increase in its prevalence.

The etiology of ASD is complex, and only 5% - 25% of the cases can be determined (6, 7). However, several genes are found to contribute to neurobehavioral disorders, including ASD, via affecting neural developments and synaptic activities (8). Multiple studies suggest that disease susceptibility may be due to SNPs in these genes. Further, diverse SNPs in each gene may lead to phenotypic variability (9).

Several susceptibility genes were found in the genome-wide study and copy number variation (CNV) study of ASD, including NLGN, SHANK, MED13, PHF3, and so on (10-12). Hence, it is widely accepted that ASD not only is a strongly heritable disease but also is related to multiple genes. Immune and inflammation dysfunctions have long been hypothesized to cause neuropsychiatric disorders, including schizophrenia, depression, and ASD (13-15). Recently, immune and inflammation-related genes have increasingly shown to be associated with ASD. Granzyme B (GZMB) is one of these genes.

GZMB is present in the granules of activated cytotoxic T cells and NK cells. Immediately after contact with target cells, GZMB enters the target cell and kills them (16). In a study, the expression of GZMB in the peripheral blood of children with ASD was found to be 2.7 times that of the con-

trol group (17).

2. Objectives

Thus, we hypothesize that GZMB might contribute to ASD development via involvement in inflammation and immune responses. However, there are a few reports of the association between GZMB and ASD. Accordingly, we selected three SNPs in GZMB, including rs2236338, rs10873219, and rs8192917, to study the association with ASD at a genetic level.

3. Methods

3.1. Ethics Statement

This study was approved by the Ethics Committee of the School of Public Health, Jilin University (permit no. 2014-05-18) in adherence to the Declaration of Helsinki guidelines. All subjects or their guardians (for children subjects) signed informed consent forms regarding the use of their samples and basic information when they were hospitalized.

3.2. Study Subjects

A case-control study was conducted in Changchun city, a northeast city of China, from June 2014 to November 2014. All subjects were enrolled from the First Hospital of Jilin University. Patients with ASD were diagnosed by Pediatric Neurology and Neurorehabilitation doctors based on the Diagnostic and Statistical Manual of Mental Disorders (fifth edition). Subjects diagnosis with schizophrenia, mania, or other psychotic disorders were excluded. Owing to the low incidence of ASD, all the diagnosed patients voluntary participated in this study. Therefore, we enrolled 268 Han population Chinese children in this case-control study, including 85 ASD patients and 183 healthy controls. Moreover, 67 nuclear family trios were also recruited in our study.

We calculated the sample size using EpiCalc 2000 software. The parameters were set as follows: 2.5 for OR (17), 80% for power, and 15% for the proportion of controls exposed (the lowest MAF of three SNPs was 0.1748). The calculated sample size was 84 for cases and 168 for healthy controls.

3.3. SNP Selection and Genotyping

According to previous research, three SNPs (rs2236338, rs10873219, and rs8192917) were selected utilizing the Haploview program (http://hapmap.ncbi.nlm.nih.gov/). We set MAF > 0.1 and $r^2 > 0.8$ for the Han Chinese population of Beijing.

We collected a 5 mL blood sample from every subject and stored the specimens at -20°C in non-anticoagulant, Plexiglas tubes. We designed polymerase chain reaction primers for these three SNPs using Assay design 3.1 (Sequenom, Inc., San Diego, CA). We extracted DNA from peripheral blood lymphocytes according to the manufacturer's instructions. The quality of the DNA was tested using an ultraviolet spectrophotometer (Beckman, Brea, CA, USA). Then, a commercial DNA extraction kit was used for genotyping the three SNPs (ClotBlood DNA kit, Catalogue number: CW0565, Beijing, China). We set up negative controls and took 10% of the samples for blind detection to avoid the bias caused by detection errors. HotStarTaq was used in PCR analysis. The conditions of PCR amplification included 15 min at 94°C for the initial cycle, followed by 45 cycles as follows: 94°C for 20 s, 56°C for 30 s, 72°C for 60 s, and finally 72°C for 10 s. Matrix-assisted laser desorption/ionization-time of flight mass spectrometry (MALDI-TOF-MS) of the MassARRAY system was used for SNP genotyping.

3.4. Statistical Analysis

The Hardy-Weinberg Equilibrium (HWE) test was applied to assess deviation in each SNP of the GZMB gene. Haploview 4.2 software was used to calculate the linkage disequilibrium coefficients. Haplotype analysis was conducted by SNP Stats program (http://bioinfo.iconcologia.net/SNPStats), and the most frequent haplotype was chosen as the reference group. The transmission disequilibrium test (TDT), a family-based association analysis, was used to evaluate the frequency of marker allele transmitted from parents (at least one is heterozygous) to the patient (18). The chi-square test and Mann-Whitney U test were conducted with IBM SPSS Statistical Software, version 24.0 (IBM Corp., Armonk. N.Y., USA).

4. Results

The age and gender distribution of the two groups in this case-control study were calculated. The median age was 4 (IQR: 3 - 5) in both healthy controls and ASD children. The male proportion was higher in the control group (80.9%) than in the case group (80.0%). However, no significant difference was found between the two groups (P = 0.960 for age; P = 0.869 for gender). All three SNPs of the GZMB gene were fitted in the HWE in both case and control groups (all P > 0.05).

As shown in Table 1, the allele distribution of the three GZMB SNPs was analyzed. Results showed no differences between the case and control groups (P = 0.787

for rs2236338, P = 0.477 for rs10873219, and P = 0.730 for rs8192917). Moreover, there was no statistical difference in genotypic frequency between ASD patients and healthy controls (all P > 0.05), even after adjustment for gender (data not shown). Based on the AIC criteria, the recessive model was the best fitting model for rs2236339 and rs8192917 and the over-dominant model for rs10873219. The associations between SNPs and ASD were not significant using these inheritance models (Table 2). In haplotype analysis, no significant difference was observed between ASD children and healthy controls (P > 0.05, Table 3).

Table 1. The Distribution of Allele and Genotype Frequency in Study Groups

SNP	Allele/Genotype	Case (%)	Control (%)	χ^2	P
rs2236338					
	G	46 (27.1)	95 (26.0)	0.073	0.787
	A	124 (72.9)	271 (74.0)		
	GG	7 (8.2)	13 (7.1)	0.112	0.946
	GA	32 (37.7)	69 (37.7)		
	AA	46 (54.1)	101 (55.2)		
rs10873219					
	T	34 (20.5)	64 (17.9)	0.506	0.477
	G	132 (79.5)	294 (82.1)		
	GG	50 (60.2)	120 (67.0)	-	0.370[a]
	GT	32 (38.6)	54 (30.2)		
	TT	1 (1.2)	5 (2.8)		
rs8192917					
	G	47 (27.6)	96 (26.2)	0.119	0.730
	A	123 (72.4)	270 (73.8)		
	GG	7 (8.2)	13 (7.1)	0.136	0.935
	GA	33 (38.8)	70 (38.3)		
	AA	45 (52.9)	100 (54.6)		

[a] P-values were calculated by Fisher's exact test.

Table 4 shows the results of TDT analysis. None of the three SNPs was significantly different between transmitted and non-transmitted alleles (χ^2 = 0.083 and P = 0.885 for rs2236338; χ^2 = 0.028 and P = 0.900 for rs10873219; and χ^2 = 0.021 and P = 0.900 for rs8192917).

5. Discussion

In our study, we not only investigated the association between three SNPs of the GZMB gene and ASD using a case-control design, but also analyzed these SNPs in 67 nuclear family trios using a family-based method, namely TDT. Unfortunately, we did not find any significant association using both approaches. Moreover, our study showed no preference for the transmission of any haplotypes.

No previous research has proven that the GZMB gene plays a critical role in developing ASD. Enstrom et al. study showed that the expression of GZMB in the peripheral blood of children with ASD is 2.7 times of the control group (17). The GZMB gene is closely related to immune cells. Brozova et al. study indicated that $CD8^+$ MAIT cells produced a high level of pro-inflammatory and cytotoxic cytokines, including granzyme B, TNF-α, and IFN-γ (19). Furthermore, it expresses more CD16, NKG2D, and CD107A after phorbol myristate acetate stimulation than another subpopulation of MAIT cells. These characteristics make $CD8^+$ MAIT cells have a better pro-inflammatory function. A study suggested that the expression of GZMB plays a pivotal role in cell contact-mediated suppression by affecting $CD4^+$ $CD25^+$ regulatory T cells (16). Moreover, Cobbold summarized that gene GZMB, along with other genes, is expressed as T-bet transfected into T cell hybridoma (20).

A few studies have revealed that ASD was associated with several immune-related genes. Torres et al. study reported that HLA and KIR immune gene alleles were significantly associated with developing ASD (21). Autistic individuals with predisposing HLA molecules bind to different aminopeptidases could induce autoantibodies (IgG, IgM or IgA) against peptides and tissue antigens (22). Several immune cell development genes, such as protooncogene (MET) and Reelin (RELN), were found to be associated with ASD (16, 23). Though we hypothesized that GZMB might play a role in ASD via affecting the immune system or neural system, the negative results in our study could not support it.

To the best of our knowledge, this is the first study to investigate the association between GZMB and ASD in the Northeast Han Chinese population. We not only conducted a case-control study but also performed a family-based study, which is less prone to false-positive results. However, limitations also exist in our study. First, our study might have biased by the relatively small sample size, affecting the statistical power to detect significant findings in the study population. Second, ASD is one of the most heritable complex disorders with many factors contributing to its development, and the mechanisms of GZMB action in ASD are barely known. Last but not least, our findings were merely based on the Northeast Han Chinese population. This could be one of the reasons for negative associations.

5.1. Conclusions

In this study, we investigated the association of granzyme B gene polymorphism with ASD using case-control and family-based methods. However, our study

Table 2. The Distribution of Genotype Frequency with the Best Fitting Inheritance Model

SNP	Genotype	Inheritance Model	Case	Control	OR (95% CI)	P
rs2236338		Recessive				0.74
	A/A-G/A		78 (91.8%)	170 (92.9%)	1.00	
	G/G		7 (8.2%)	13 (7.1%)	0.85 (0.33 - 2.22)	
rs10873219		Over-dominant				0.18
	G/G-T/T		51 (61.5%)	125 (69.8%)	1.00	
	G/T		32 (38.5%)	54 (30.2%)	0.69 (0.40 - 1.19)	
rs8192917		Recessive				0.74
	A/A-G/A		78 (91.8%)	170 (92.9%)	1.00	
	G/G		7 (8.2%)	13 (7.1%)	0.85 (0.33 - 2.22)	

Table 3. Haplotype Analysis of GZMB in ASD Children and Healthy Controls

	rs2236338	rs10873219	rs8192917	Frequency			OR (95% CI)	P
				Total	Case	Control		
1	A	G	A	0.7272	0.7171	0.7319	1.00	-
2	G	T	G	0.1796	0.2009	0.17	0.81 (0.50 - 1.32)	0.41
3	G	G	G	0.0816	0.0697	0.0868	1.20 (0.61 - 2.38)	0.59
Rare	*	*	*	0.0116	0.0123	0.0113	0.89 (0.16 - 5.01)	0.89

Table 4. TDT Analysis for the SNPs Association with ASD

SNP	Allele	Transmitted	χ^2	P
rs2236338			0.083	0.885
	G	25		
	A	23		
rs10873219			0.028	0.900
	G	18		
	T	17		
rs8192917			0.021	0.900
	G	24		
	A	23		

could not prove the association between GZMB and ASD, or its pathogenetic mechanisms in ASD development. Diverse ethnics and larger sample sizes are required to test our hypothesis in further research.

Footnotes

Authors' Contribution: Study concept and design: Qiong Yu. Analysis and interpretation of data: Guojun Kang and Shangchao Zhang. Drafting of the manuscript: Guojun Kang. Critical revision of the manuscript for important intellectual content: Qiong Yu. Statistical analysis: Guojun Kang and Shangchao Zhang. Administrative, technical, and material support: Yaqin Yu and Qiong Yu.

Conflict of Interests: We declare that there is no conflict of interest in this paper.

Ethical Approval: This study was approved by the Ethics Committee of the School of Public Health, Jilin University (permit no. 2014-05-18), in adherence to the Declaration of Helsinki guidelines.

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Patient Consent: All subjects or their guardians (for children subjects) signed informed consent forms regarding the use of their samples and basic information when they were hospitalized.

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