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Family-based association study of *ZNF533*, *DOCK4* and *IMMP2L* gene polymorphisms linked to autism in a northeastern Chinese Han population*

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Abstract: Objective: A study in a Caucasian population has identified two single-nucleotide polymorphisms (SNPs) in *ZNF533*, one in *DOCK4*, and two in *IMMP2L*, which were all significantly associated with autism. They are located in *AUTS1* and *AUTS5*, which have been identified as autism susceptibility loci in several genome-wide screens. The present study aimed to investigate whether *ZNF533*, *DOCK4*, and *IMMP2L* genes are also associated with autism in a northeastern Chinese Han population. Methods: We performed a similar association study using families with three individuals (one autistic child and two unaffected parents). A family-based transmission disequilibrium test (TDT) was used to analyze the results. Results: There were significant associations between autism and the two SNPs of *ZNF533* gene (rs11885327: χ^2=4.5200, P=0.0335; rs1964081: χ^2=4.2610, P=0.0390) and the SNP of *DOCK4* gene (rs2217262: χ^2=5.3430, P=0.0208). Conclusions: Our data suggest that *ZNF533* and *DOCK4* genes are linked to a predisposition to autism in the northeastern Chinese Han population.

Key words: Autism, *ZNF533*, *DOCK4*, *IMMP2L*, Northeastern Chinese Han population, Single-nucleotide polymorphism
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1 Introduction

Autism (OMIM: 209850) is a highly heritable neurodevelopmental disorder that first appears in childhood and belongs to the group of autism spectrum disorders (ASDs). Revised diagnostic criteria for ASDs, recently published in the latest Diagnostic and Statistical Manual of Mental Disorders, 5th Ed. (DSM-V), include two core areas: communication and social deficits, and fixed or repetitive behaviors (American Psychiatric Association, 2013). Epidemiological studies estimate that the prevalence of ASD has increased substantially, from 0.04% to 0.05% of the population in the 1960s to about 0.62% currently (Baird *et al.*, 2006; Fombonne, 2009; Kogan *et al.*, 2009; Brugha *et al.*, 2011; Kim *et al.*, 2011; Elsabbagh *et al.*, 2012). Recently, the prevalence of ASD has been estimated at 1/88 in the USA, and about 1/54 boys and 1/252 girls (Autism and Developmental Disabilities Monitoring Network Surveillance Year 2008 Principal Investigators, 2012). This remarkable increase in prevalence has received considerable attention, with concomitant increases in research on etiology.

Although the pathogenesis of autism remains unclear, previous investigations have found that it involves genetic and environmental factors. Studies on families and twins have indicated that genetic factors play an important role in the etiology of autism, and have estimated the heritability of autism to be more than 90% (Bailey *et al.*, 1995; Hallmayer *et*

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al., 2011). The genetic background of autism is complex and involves multiple genes with significant functions in neurodevelopment (Trikalinos *et al.*, 2006; Xu *et al.*, 2012). The International Molecular Genetic Study of Autism Consortium (IMGSAC) has identified two autism linkage loci on chromosomes 7q21-q32 (designated as autism susceptibility locus 1, *AUTS1*) (IMGSAC, 1998) and 2q24-q33 (designated as *AUTS5*) (IMGSAC, 2001). Meta-analyses (Badner and Gershon, 2002; Trikalinos *et al.*, 2006), follow-up studies (Buxbaum *et al.*, 2001; Shao *et al.*, 2002a), and at least four genome-wide screens have confirmed that these two loci are linked to autism (IMGSAC, 1998; Barrett *et al.*, 1999; Shao *et al.*, 2002b; Schellenberg *et al.*, 2006).

Maestrini *et al.* (2010) performed high density single-nucleotide polymorphism (SNP) mapping in *AUTS1* and *AUTS5*, using family-based and case-control association analysis. They found that several SNPs, from *ZNF533* (zinc finger protein 533), *IMMP2L* (IMP2 inner mitochondrial membrane protease-like), and *DOCK4* (dedicator of cytokinesis 4), were associated with the susceptibility of the pathogenesis of autism in a Caucasian population. In *ZNF533*, they found, by TDT analysis, that rs11885327 ($P<8.0\times10^{-4}$) and rs1964081 ($P<1.4\times10^{-3}$) were strongly related to autism. In *DOCK4*, rs2217262 ($P<9.2\times10^{-4}$) was the most significant locus associated with autism by family-based analysis of the two independently cohorts. In *IMMP2L*, rs1528039 ($P<1.2\times10^{-4}$) and rs12537269 ($P<6.3\times10^{-4}$) were the candidate loci for the development of autism in Caucasians. Because of differences in genetic background, the causative susceptibility SNPs for a given disease are not necessarily consistent across populations. To understand better the pathogenetic mechanism of autism in Chinese populations, in this study we investigated the association between autism and SNPs of *ZNF533* (rs11885327, rs1964081), *DOCK4* (rs2217262), and *IMMP2L* (rs12537269, rs1528039) in the northeastern Chinese Han population using a family-based association study.

2 Materials and methods

2.1 Subjects

This study examined 370 self-identified Chinese Han families-of-three residing in northeastern China. Each family comprised a singleton autistic disorder patient and unaffected biological parents (317 males, 53 females). Owing to the "Chinese Family Planning Policy" in China, all families involved in this experiment had the same composition: each family had only three members (father-mother-singleton child). Patients were recruited from the Children Development and Behavior Research Center (CDBRC), Harbin Medical University, Heilongjiang Province, China, during January 2007 to September 2011. The mean age of the children was 7.77 years (range 3–19 years). More than two experienced psychiatrists independently issued autism diagnoses according to the international Diagnostic and Statistical Manual of Mental Disorders, 4th Ed. (DSM-IV) criteria (American Psychiatric Association, 1994), which characterized autism by impaired social relationships and communication, language deficits or disorders, and restricted, repetitive, or stereotyped behaviors. The cases were also assessed using the Childhood Autism Rating Scale (CARS) (Schopler *et al.*, 1980) and the Autism Behavior Checklist (ABC) (Krug *et al.*, 1980). Cases with Rett syndrome, tuberous sclerosis, fragile-X syndrome, and any other neurological conditions suspected to be associated with autism, were excluded by clinical examination and molecular genetic tests of the *FMR1* gene (Khaniani *et al.*, 2008). Approval from the Ethics Committee of Harbin Medical University was obtained before initiating the study. According to the Declaration of Helsinki, parents or guardians of all participants provided written informed consent for children to participate in the study after the study procedures had been explained to them [HMUIRB20120015].

2.2 Selection of candidate SNPs, DNA extraction and genotyping

To be eligible for this study, SNPs were required to meet all of the following inclusion criteria: (1) must have a strong correlation with autism in populations other than Chinese, such as Caucasian; (2) based on the National Center for Biotechnology Information (NCBI) database, must be found at moderate to high frequencies (minor allele frequency (MAF)≥0.05) among the population to ensure attainable detection; and (3) must not have been reported previously to have an association with autism in Chinese populations. According to these criteria, we selected five

SNPs in the regions of *AUTS1* and *AUTS5* for our genotyping study: rs11885327 and rs1964081 for *ZNF533*, rs2217262 for *DOCK4*, and rs12537269 and rs1528039 for *IMMP2L*.

Blood samples were obtained from the autistic patients and their parents. Genomic DNA was extracted from whole blood using Qiagen QIAamp DNA mini kits. All DNA samples were stored at −86 °C. A 4-μg aliquot of each genomic DNA sample was dispensed in a bar-coded 96-well microtiter plate at a concentration of 100 ng/μl and genotyped using a SNaPshot Multiplex kit (Applied Biosystems, Foster City, CA, USA).

The five SNPs were assembled for SNaPshot genotyping into eight small multiplexes. Primers to amplify a different sized fragment for each SNP within a multiplex were designed using Primer 5.0 (http://frodo.wi.mit.edu/). Extension primers, also amplifying different length fragments within a multiplex, were picked from the sequence immediately upstream or downstream of the SNP. The information on primers is presented in Table 1.

The polymerase chain reaction (PCR) had a total reaction volume of 40 μl, containing 10× PCR buffer (Mg^{2+} free), $MgCl_2$ (50 mmol/L), dNTP (10 mmol/L), 4 pmol/L of each primer, platinum Taq (5 U) DNA polymerase, and 50 ng of genomic DNA. The PCR amplification conditions were as follows: denaturation at 94 °C for 2 min followed by 6 cycles at 94 °C for 20 s, annealing at 55 °C for 30 s, and extension at 72 °C for 45 s. Subsequently, the annealing temperature was decreased by 1 °C every two cycles, which was followed by 5 cycles of denaturation at 94 °C for 30 s, annealing at 53 °C for 30 s, and extension at 72 °C for 40 s, and then 25 cycles of denaturation at 94 °C for 30 s, annealing at 52 °C for 30 s, and extension at 72 °C for 40 s, and final extension at 72 °C for 5 min. PCR products were treated with Exonuclease I (ExoI, 20 U; Fermentas) and Shrimp Alkaline Phosphatase (SAP, 1 U; Fermentas), and incubated at 37 °C for 1 h followed by a 75 °C incubation for 15 min and 4 °C for 24 h. The extension reaction included 1× ABI Prism SNaPshot Multiplex ready reaction mix (Applied Biosystems), 0.4 mmol/L of each primer, and 1.5 μl of each PCR product. PCR was conducted according to the manufacturer's recommendations (Applied Biosystems). The extension PCR products were treated with ExoI and SAP and then run on a genetic analyzer (ABI 3730; Applied Biosystems).

Data were viewed and analyzed using the software GeneMapper v4.1 (Applied Biosystems). Because peak heights in heterozygotes are not always equal, we chose a threshold to distinguish between heterozygotes and homozygotes. Using the following equation (allele 1 peak height−allele 2 peak height)/(allele 1 peak height+allele 2 peak height) to calculate a threshold, we formulated a Perl script in-house to assign a value between +1 and −1 for each sample. We then inspected the distribution of values from each SNP for all samples and set an appropriate threshold.

2.3 Statistical analysis

The Hardy-Weinberg equilibrium for genotype frequencies was estimated using the chi-square test (significance level of 0.05). The MAF was ≥5% in all samples. The call rate of each SNP was ≥95%. We performed a family-based transmission disequilibrium

Table 1 PCR primers used in SNaPshot analysis

Gene	SNP	Primer sequence	Product (bp)
ZNF533	rs11885327	Forward: 5'-TGCCACCATCTTGGTTCTGA-3'	627
		Reverse: 5'-CAAAAGCAGATCGAGCACCTAC-3'	
	rs1964081	Forward: 5'-GATGTGTAAAGCACAAATGCAGTA-3'	337
		Reverse: 5'-CTGGGCCTCTGTTGCTCATT-3'	
DOCK4	rs2217262	Forward: 5'-ACTGAGCCTCGGCTTCTTCAT-3'	496
		Reverse: 5'-TAGCGCCTCCTTACACAGGAA-3'	
IMMP2L	rs1528039	Forward: 5'-ATGGGCCATAAAAACCATTAAC-3'	188
		Reverse: 5'-TCTCCACCAGCCTTCCGTAT-3'	
	rs12537269	Forward: 5'-GGGCTGGGAGCACTTATAGAA-3'	205
		Reverse: 5'-CACCAGATACGCTCTCCACATT-3'	

test (TDT) (Spielman *et al.*, 1993; Spielman and Ewens, 1996) by comparing the preferential allelic transmission from heterozygous parents to affected offspring. Haploview v4.2 was used to perform the analysis described above. Haploview is commonly used bioinformatics software which is designed to analyze and visualize patterns of linkage disequilibrium in genetic data. A two-tailed $P<0.05$ was inferred as statistically significant.

3 Results

All 370 families were genotyped for the five autism-associated SNPs (rs11885327, rs1964081, rs2217262, rs12537269, and rs1528039; Table 2) using an ABI Prism SNaPshot Multiplex kit (Applied Biosystems). Genotype frequencies were found to be in Hardy-Weinberg equilibrium (P=0.5748, 0.6379, 0.1368, 0.3857, and 0.7995, respectively). All of these SNPs were polymorphic and were used subsequently as genetic markers for the association study. Four families were excluded from further analyses because they did not conform to a Mendelian inheritance pattern, leaving 366 families involved in this study.

For rs11885327 in the *ZNF533* gene, the T allele frequencies were 57.8% for autistic patients and 55.1% for their unaffected parents. The G allele frequencies for rs1964081 were 56.3% for patients and 53.6% for parents. In the *DOCK4* gene, the A allele frequencies for rs2217262 were 93.7% for patients and 92.1% for parents. For rs1528039 in the *IMMP2L* gene, the G allele frequencies were 15.2% for patients and 14.5% for parents. Likewise, the A allele frequencies of rs12537269 were 15.2% for patients and 14.5% for parents. All frequencies of alleles and genotypes are presented in Table 2.

The TDT analysis showed that alleles T and G of rs11885327 and rs1964081, which are the polymorphisms of the *ZNF533* gene, would be preferentially transmitted to the affected offspring (χ^2=4.5200, P=0.0335; χ^2=4.2610, P=0.0390, respectively). For rs2217262, which is the polymorphism of the *DOCK4* gene, allele A would be preferentially transmitted to the affected offspring (χ^2=5.3430, P=0.0208). For the polymorphisms of the *IMMP2L* gene, the preferential transmission alleles were G for rs1528039 (χ^2=0.4380, P=0.5082) and A for rs12537269 (χ^2=0.4290, P=0.5127). These three SNPs (rs11885327, rs1964081, and rs2217262) showed evidence of transmission disequilibria and were associated with autism (Table 3).

Table 2 Allele and genotype frequencies of five SNPs in 366 autism trios

Gene	SNP	Allele and genotype frequency*	
		Patient	Parent
ZNF533	rs11885327		
	T	423 (57.8%)	806 (55.1%)
	C	309 (42.2%)	658 (44.9%)
	TT	122 (33.3%)	226 (30.9%)
	TC	179 (48.9%)	354 (48.4%)
	CC	65 (17.8%)	152 (20.8%)
	rs1964081		
	A	320 (43.7%)	679 (46.4%)
	G	412 (56.3%)	785 (53.6%)
	AA	65 (17.8%)	161 (22.0%)
	AG	190 (51.9%)	357 (48.8%)
	GG	111 (30.3%)	214 (29.2%)
DOCK4	rs2217262		
	A	686 (93.7%)	1349 (92.1%)
	C	46 (6.3%)	115 (7.9%)
	AA	323 (88.3%)	625 (85.4%)
	AC	40 (10.9%)	99 (13.5%)
	CC	3 (0.8%)	8 (1.1%)
IMMP2L	rs1528039		
	A	621 (84.8%)	1251 (85.5%)
	G	111 (15.2%)	213 (14.5%)
	AA	262 (71.6%)	533 (72.8%)
	AG	97 (26.5%)	185 (25.3%)
	GG	7 (1.9%)	14 (1.9%)
	rs12537269		
	A	111 (15.2%)	213 (14.5%)
	G	621 (84.8%)	1251 (85.5%)
	AA	7 (1.9%)	12 (1.6%)
	AG	97 (26.5%)	189 (25.8%)
	GG	262 (71.6%)	531 (72.5%)

* Data are expressed as number (percentage)

4 Discussion

Autism is a highly heritable complex disorder, and the pathogenesis is unclear. Maestrini *et al.* (2010) found that two SNPs (rs11885327 and rs1964081) in *ZNF533* gene, one (rs2217262) in *DOCK4* gene,

Table 3 Transmission disequilibrium test (TDT) analysis of allelic association between SNPs and autism

Gene	SNP	Allele	T[a]	NT[a]	Chi-square	*P*-value
ZNF533	rs11885327	T	197	157	4.5200	0.0335
		C	157	197		
	rs1964081	A	159	198	4.2610	0.0390
		G	198	159		
DOCK4	rs2217262	A	61	38	5.3430	0.0208
		C	38	61		
IMMP2L	rs1528039	A	88	97	0.4380	0.5082
		G	97	88		
	rs12537269	A	99	90	0.4290	0.5127
		G	90	99		

[a] T and NT respectively stand for times each allele was transmitted and not transmitted

and two (rs12537269 and rs1528039) in *IMMP2L* gene were significantly associated with autism in a Caucasian population. However, the roles of these SNPs in the development of autism in Chinese populations are poorly understood.

In the present family-based study, we investigated the allele frequencies of these five SNPs (rs11885327, rs1964081, rs2217262, rs1528039, and rs12537269) in autism patients and their parents, and analyzed the association between these loci and autism in a northeastern Chinese Han population. We found that three SNPs (rs11885327, rs1964081, and rs2217262) were significantly associated with autism (P=0.0335, 0.0390, and 0.0208, respectively). The polymorphisms rs11885327 and rs1964081 in *ZNF533* and rs2217262 in *DOCK4* were significantly associated with autism not only in the Caucasian population but also in the Chinese Han population. In contrast, the polymorphisms rs1528039 and rs12537269 in *IMMP2L* were not associated with autism in the Chinese Han population. This discrepancy indicates that population substructure could differentially affect the predisposition of populations to a specific disease. Our findings provided additional evidence that chromosomal regions 2q31.2-q31.3 and 7q31-q31.1 are plausible candidates for autism. Two genes (*ZNF533* and *DOCK4*) located in these regions have been recognized to affect neural development, and may play an important role in the etiology of autism.

ZNF533 is located in the *AUTS5* region (2q31.2-q31.3) and encodes a highly conserved protein that contains 4 matrin-type zinc fingers. *ZNF533* has been identified as a putative repressor of transcription. It is widely expressed in adult brain tissues and is involved in the development of the palate and lip (Beaty *et al.*, 2006). Several studies have shown that deletions of the *ZNF533* gene are associated with mental retardation (Kleefstra *et al.*, 2004; Monfort *et al.*, 2008). In addition, the expression of all isoforms of this gene in the fetal brain has been confirmed using reverse transcription (RT)-PCR (Maestrini *et al.*, 2010). In our study, we found that the polymorphisms rs11885327 and rs1964081, located within the introns of *ZNF533*, were associated with autism in a northeastern Chinese Han population. A significant association was also observed between these two SNPs and autism in a family-based association study of a Caucasian population (Maestrini *et al.*, 2010). Taken together, these studies support a role for these SNPs in *ZNF533* in the development of autism.

DOCK4 is located in the *AUTS1* (7q31.1) region and encodes a protein involved in intracellular signaling networks. Animal studies have shown that *DOCK4* deficiency reduces dendritic growth and branching in hippocampal neurons (Miyamoto and Yamauchi, 2010). Previous studies have revealed that *DOCK4* is widely expressed in many tissues, including the brain and nervous system (Miyamoto and Yamauchi, 2010), and plays a role in axonal patterning in the embryonic central nervous system (CNS) (Biersmith *et al.*, 2011). Mutations in the *DOCK4* gene are associated with ovarian, prostate, and colorectal cancers, as well as glioma. Previous studies have also shown that *DOCK4* is associated with Tourette's syndrome (Díaz-Anzaldúa *et al.*, 2004) and cytogenetic aberrations (Petek *et al.*, 2001). Genomic disruption of *DOCK4* results in an additive effect and can lead to a more severe autism spectrum

phenotype (Pagnamenta *et al.*, 2010). Xiao *et al.* (2013) have demonstrated an important role of *DOCK4* in neurite differentiation during early neuronal development. A fine-mapping study of *AUTS1* found that the rs2217262 polymorphism located within the *DOCK4* gene was an autism susceptibility factor (Maestrini *et al.*, 2010). In the current study, we also found that rs2217262 was significantly associated with autism and detected a preferential transmission of the A allele to the affected offspring (χ^2=5.3430, P=0.0208). Therefore, based on previous studies and the current results, the rs2217262 SNP of the *DOCK4* gene is likely to be associated with autism in the Caucasian and northeastern Chinese Han populations.

The third candidate gene involved in autism, *IMMP2L*, is located in the *AUTS1* region (7q31) and encodes an inner mitochondrial membrane protease-like protein. A disruption in the *IMMP2L* gene was found in an individual with Tourette's syndrome, a complex neuropsychiatric disorder (Petek *et al.*, 2001). *IMMP2L* partially encompasses the neuronal leucine-rich repeat gene 3 (LRRN3), which is highly expressed in the fetal brain. Animal studies have demonstrated that the members of the LRR family play an essential role in target recognition, axonal pathfinding, and cell differentiation during neural development (Battye *et al.*, 2001). Previous studies have shown that the rs1528039 and rs12537269 polymorphisms of *IMMP2L* are related to autism in Caucasians (Maestrini *et al.*, 2010). In our current study, however, we found no association between these two SNPs and autism in the northeastern Chinese Han population. Genetic heterogeneity and phenotypic heterogeneity are possible explanations for the differences between the two studies. Further, Petek *et al.* (2007) reported no deleterious mutations in *IMMPL2* in ASD. Together, these studies suggest that the different findings may reflect differences in race, disease sensitivity and severity, even in the same population.

What reasons might account for the discrepancies regarding different disease 'risk' alleles? First, genetic heterogeneity and phenotypic heterogeneity could contribute to the different genetic associations. Many previous investigations focused on ASD as a whole, but not solely on autism, which may have been a confounding factor. Some genetic studies examined autism separately from ASD (Szatmari *et al.*, 2007; Zhou *et al.*, 2011) to limit the problem of phenotypic heterogeneity somewhat. In this study, we selected subjects with a confirmed diagnosis of autism, so as to reduce heterogeneity and to make our observations more accurate. In addition, the TDT study with three individuals in a family allowed us to consider possible confounding effects induced by population stratification. Furthermore, different genetic backgrounds play an important role in disease susceptibility. The ethnic differences between the Caucasian and Chinese populations, and the sample size of the homogeneous ethnic group studies may account for the differences observed between the two studies.

In summary, in a family-based association study of a northeastern Chinese Han population, we identified three SNPs (rs11885327, rs1964081, and rs2217262) associated with autism. In further studies, it will be interesting to verify these findings in populations with different ethnicities and to determine their functional contribution to autism.

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Compliance with ethics guidelines

Shuang LIANG, Xue-lai WANG, Ming-yang ZOU, Han WANG, Xue ZHOU, Cai-hong SUN, Wei XIA, Li-jie WU, Takashi X. FUJISAWA, and Akemi TOMODA declare that they have no conflict of interest.

All procedures followed were in accordance with the ethical standards of the responsible committee on human experimentation (institutional and national) and with the Helsinki Declaration of 1975, as revised in 2008 (5). Informed consent was obtained from all patients for being included in the study. Additional informed consent was obtained from all patients for which identifying information is included in this article.

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中文概要：

本文题目：*ZNF533*、*DOCK4* 和 *IMMP2L* 基因多态性与中国东北汉族孤独症的关联研究

Family-based association study of *ZNF533*, *DOCK4* and *IMMP2L* gene polymorphisms linked to autism in a northeastern Chinese Han population

研究目的： *ZNF533*、*DOCK4* 和 *IMMP2L* 在大脑发育过程中起到非常重要的作用，是孤独症研究的候选基因。高加索人群的研究结果发现，*ZNF533*、*DOCK4* 和 *IMMP2L* 基因的 5 个单核苷酸多态性（SNP）位点与孤独症高度相关。为了探讨上述位点是否与中国孤独症发生相关，我们开展了东北汉族孤独症的核心家系研究。

创新要点： 孤独症候选基因多态位点的研究结果通常很难得到重复。本研究首次在中国东北汉族人群中验证了与高加索人群孤独症密切相关的候选位点。这一结果对孤独症的研究具有重要指导意义。

研究方法： 利用 SNaPshot 的方法，检测了中国东北汉族 370 个核心家系中 *ZNF533*（rs11885327、rs1964081）、*DOCK4*（rs2217262）和 *IMMP2L*（rs12537269、rs1528039）的分布情况，利用传递不平衡检验（TDT）分析了这些多态位点与孤独症发生的相关性。

重要结论： *ZNF533* 和 *DOCK4* 基因多态性与中国北方汉族孤独症发生存在显著关联。

关键词组： 孤独症；*ZNF533*；*DOCK4*；*IMMP2L*；中国东北汉族；单核苷酸多态性