Original Article
Association between NKX2-5 rs29784 and infantile hypertrophic pyloric stenosis in Chinese Han population

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Abstract: Purpose: The aim of this study was to evaluate the association of three single nucleotide polymorphisms (SNPs, rs11712066 and rs573872 near MBNL1, rs29784 near NKX2-5) with infantile hypertrophic pyloric stenosis (IHPS) in Chinese Han population. Methods: A total of 47 family trios consisting of infants with IHPS and their healthy biological parents were recruited for this study. Genotypes were determined using direct sequencing. Transmission disequilibrium test (TDT) was performed for family-based association analysis. Results: Genotypic distributions of three SNPs in both groups (patients and proband's parents) were in conformity with Hardy-Weinberg equilibrium (P > 0.05). There were significant preferential transmission of A allele of rs29784 from the parents to affected offspring (TDT: x^2 = 5.444, P = 0.0196). However, other two polymorphism loci (rs11712066 and rs573872) were not significant susceptibility loci for IHPS in Chinese Han population. Conclusions: We found that there was a significant association between rs29784 and IHPS.

Keywords: Infantile hypertrophic pyloric stenosis, transmission disequilibrium test, single nucleotide polymorphism, rs29784

Introduction

Infantile hypertrophic pyloric stenosis (IHPS) is one of the most common congenital defects. Many studies demonstrate that the male predominance (male: female ratio of 4-5:1) [1-6] and the incidence rate is about 1-8 per 1,000 live births varying with the race and geography [7-11]. IHPS is a common cause of gastric outlet obstruction in infant, which results from hypertrophy and thickening of the circular muscle of the pylorus. It is well known that both environmental and genetic factors contribute to the development of IHPS, although its pathogenetic mechanism is still unclear [12, 13]. Therefore, identification of genetic factors may be useful for the elucidation of underlying pathological mechanism of IHPS.

Some loci associated with IHPS have been reported (**Table 1**). Recently, three single nucleotide polymorphisms (SNPs) (rs11712066, rs573872 and rs29784) have been identified to

be associated with IHPS in Denmark through genome-wide linkage analysis and re-sequencing by Feenstra et al [14]. And two loci (rs11712066 and rs29784) were confirmed to be associated with IHPS in European (UK and Ireland) by Everett [15].

However, there have been no reports on the associations of rs11712066, rs573872, rs29784 with IHPS in Chinese Han population. The aim of this study was to evaluate the association between rs11712066, rs573872, rs29784 and IHPS in Chinese Han population.

Materials and methods

Subjects

A total of 47 family trios (one affected offspring and two healthy biological parents) were recruited between July 2007 and February 2014 in Guangzhou First People's Hospital. All subjects were Han Chinese. The diagnosis of IHPS was based on a history of projectile vomiting, palpa-

Table 1. Genetic studies on IHPS

References	Type	Ethnicity	Sample size	IHPS susceptibility loci
Chung et al [16]	Linkage analysis	Caucasian origin	27 families (229 individuals, 87 with IHPS)	*NOS1* (12q12.2-q24.31)
Capon et al [17]	Linkage analysis	northern European families	3-generation family (10 affected)	16p12-p13
Everett et al [18]	Linkage analysis	UK and Ireland	81 families (302 individuals, 206 with IHPS)	11q14-q22 and Xq23i
Everett et al [19]	Linkage analysis	Caucasian	1 family (8 with IHPS)	16q24
Saur et al [20]	Association study	Belgium	Case control, 16 IHPS and 81 controls	-84 (GNA) of *nNOS* has been identified to be associated with IHPS
Bjarke Feenstra [14]	genome-wide association study	Denmark	1,001 cases and 2,401 controls	*MBNL1* and *NKX2-5*
Everett KV [15]	Intrafamilial association analysis	northern European	301 trios as well as in a further 16 nuclear pedigrees	*MBNL1* and *NKX2-5*

Table 2. Demographic and clinical characteristics of the patients

Male/female	42/5
Birth weight (kilograms)	3.10±0.46 (1.90~4.20)
Symptom onset age (days)	22.8±10.1 (7~50)
Pyloric muscle long (cm)	1.90±0.27 (1.45~2.70)
Pyloric muscle thickness (cm)	0.47±0.10 (0.3~0.76)

tion of an olive mass, ultrasonography, and upper gastrointestinal brium series. There were 42 males and 5 females, and the clinical features were listed in **Table 2**. Written Informed consent was obtained from each infant's legal guardian before peripheral blood samples collection. The study was approved by the Ethics Committee of Guangzhou First People's Hospital.

DNA sequence analysis

After finishing the clinical examination, the rest blood for each infant was collected and stored at -80°C. Furthermore, 2 ml of peripheral blood samples were collected from the infants' parents. The genomic DNA was extracted using QIAamp® DNA Blood kit (Qiagen, Germany) according to the manufacturer's instructions. The concentration and quality of genomic DNA were measured by Nanodrop 1000, and then stored at -80°C for further use.

All samples were genotyped using polymerase chain reaction (PCR) and direct sequencing. The PCR amplification was performed using Ta-KaRa Ex Taq® (TaKaRa, Dalian, China) in a final volume of 25 μl, containing 2.5 μl Takara 10× Ex Taq Buffer (Mg^{2+} plus), 2 μl dNTP Mix (2.5 mM each), 0.25 μl Takara *Ex Taq* DNA polymerase (5 U/μl), 0.4 μM of each primer, and 100 ng of the genomic DNA.

The amplification program for *rs11712066* (Forward primer: 5'-AAATCCTGAGCCCTCCATG-3'; reverse: 5'-TAGGCTTTGCTCTTGATAGTGATG-3', product size: 653 bp) included an initial denaturation at 94°C for 5 min, followed by 30 cycles of denaturation at 94°C for 1 min, annealing at 58°C for 1 min, and extension at 72°C for 1 min and then once at 72°C for 10 min. The conditions for *rs573872* (Forward primer: 5'-CACA-AAGCTCGGCCATACAC-3'; reverse: 5'-CTCTTCA-CAGGGCAGCAGGA-3', product size:463 bp) included an initial denaturation at 94°C for 5 min, followed by 40 cycles of denaturation at

94°C for 30 s, annealing at 62°C for 30 s, and extension at 72°C for 90 s and then once at 72°C for 10 min. The reaction for *rs29784* (Forward primer: 5'-AGACCACCATTAGCCAAG-GA-3'; reverse: 5'-CGTGGCAAGAGGTGAGTGT-3', product size:676 bp) was carried out in the following conditions: an initial melting step of 4 min at 94°C, followed by 30 cycles of 30 s at 94°C, 40 s at 58°C, and 30 s at 72°C, and a final elongation step of 10 min at 72°C.

PCR products were purified using a QIAQuick PCR purification kit (Qiagen, German). Direct sequencing of the samples was performed on the ABI 3730xl DNA Analyzer using a BigDye Terminator kit v3.1 (Applied Biosystems) with the corresponding forward primer as the sequencing primer. Sequencing results were compared with the reference human sequence.

Statistical analysis

The observed genotype frequencies in cases and controls were tested for Hardy-Weinberg equilibrium using an online calculator (http://www.oege.org/software/hwe-mr-calc.shtml). The transmission disequilibrium test (TDT) was applied to analyze the family-based genotyping data. The minor allele frequency (MAF) and TDT analysis were performed using Haploview software (version 4.2). A value of *P* less than 0.05 was considered statistically significant.

Results

We found *rs11712066* was not polymorphic, so it was excluded from further analysis. The observed genotype frequencies of *rs573872* and *rs29784* in patients and their parents were not different from those expected from the Hardy-Weinberg equilibrium (*P* > 0.05) (**Table 3**). We detected significant preferential transmission in *rs29784* in the TDT analysis using haploview software (version 4.2), the results demonstrated the preferential transmission of the A allele compared to the G allele (TDT x^2 = 5.444, *P* = 0.0196) in IHPS (**Table 4**).

Discussion

Up to date, there were numerous studies have been conducted to identify the susceptibility loci for IHPS such as *NOS1*, *TRPC6*, *molitin*, etc [16-20]. Recently, Feenstra et al [14] conducted a genome-wide association study (GWAS) on 1,001 IHPS cases and 2,401 controls, and found three SNPs (*rs11712066* (located 150

Table 3. Results of Hardy-Weinberg equilibrium test

Marker ID	Genotype	case	MAF	X^2	P-value	parents	MAF	X^2	P-value
rs573872	TT/TG/GG	27/17/3	0.245	0.02	> 0.05	60/30/4	0.202	0.01	> 0.05
rs29784	AA/AG/GG	3/24/20	0.319	1.44	> 0.05	7/35/52	0.261	0.11	> 0.05

Table 4. TDT results for the SNPs

SNPs	Overtransmitted allele	transmitted	Not-transmitted	TDT X^2	P value
rs29784	A	25	11	5.444	0.0196
rs573872	G	18	12	1.2	0.2733

T: Transmitted, NT: non-transmitted.

kb upstream of *MBNL1*), *rs573872* (approximately 1.3 Mb downstream of *MBNL1*), *rs-29784* (64 kb downstream of *NKX2-5*) associated with IHPS. Two of these three SNPs (*rs-11712066* and *rs29784*) were confirmed by Everett [15] through replication analysis. So *NKX2-5* and *MBNL1* were considered the most likely functional candidates gene for IHPS.

NKX2-5 encodes the *NK2* homeobox 5 transcription factor, which has been shown to have a critical role in normal heart development [21-24]. *Nkx2-5* is independently required for the development of a pyloric outer longitudinal muscle fascicle, which is required for pyloric sphincter morphogenesis in mice. Alter *Nkx2-5* expression maybe contribute to pathogenesis of infantile hypertrophic pyloric stenosis [25, 26], indicating that *Nkx2.5* appear to be induce pyloric-specific epithelial morphology, and hence play at least partially redundant functions in formation of the pyloric sphincter [27].

Human *MBNL1* is a double-stranded RNA-binding protein, specificlly binding to *CUGexp* RNA positively correlates with repeat length [28]. *MBNL1* promotes skipping of cTNT exon 5 by binding directly to the upstream intron [29], and the *MBNL1*-binding site on this intron forms a hairpin structure containing mismatches similar to the expanded *CUG* repeats [30, 31]. *MBNL1* has been implicated in the regulation of RNA transcription, processing and stability of downstream targets, possibly involved in DM pathogenesis [32]. So *MBNL1* is considered to be a regulator for alternative splicing [33]. *MBNL1* regulates a subset of splicing events during postnatal development [34]. Especially for postnatal skeletal muscle development, and the activity of Human *MBNL1* is controlled by a switch its localization from cyto-

plasmic to predominantly nuclear [35]. Thus *MBNL1* appears to be involved in muscle differentiation.

The results of our present study showed that *rs29784* was associated with IHPS, while other two SNPs had negative association with IHPS in study population. Although in our study we did not find any significant association between *MBNL1* SNPs (*rs573872, rs11712066*) and IHPS, the above mentioned bulk of literature, in showing *MBNL1* involving in muscle differentiation, suggesting *MBNL1* maybe a susceptible gene with IHPS.

In conclusion, our replication study indicated that there was association between *rs29784* and IHPS in Chinese Han population. And *NKX2-5* may play a role in the pathogenesis of IHPS. The association between the two SNPs (*rs573872* and *rs11712066*) and IHPS was negative and it still requires further investigations in different ethnic groups with larger samples and functional analysis.

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Disclosure of conflict of interest

None.

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