

**TITLE: Lack of association between *nNOS* -84G>A polymorphism and risk of infantile hypertrophic pyloric stenosis in a Chinese population**

**SHORT TITLE:** *nNOS* in IHPS disease

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**ABSTRACT**

**Background:** Infantile hypertrophic pyloric stenosis (IHPS) is one of the most common gastrointestinal obstructions in the infancy requiring surgery. Reduced-expression of *nNOS*, which plays an important role in the regulation of the human pyloric muscle, is thought to underlie IHPS. The role of *nNOS* in IHPS has been supported by the genetic association of a functional regulatory *nNOS* polymorphism (-84A>G) with IHPS in Caucasians. We reasoned that the corroboration of this association in a population of different ethnic origin would prompt follow-up studies and further investigation of the IHPS pathology at molecular level. Thus, we attempted to reproduce the original findings in a Chinese population of comparable size in what would be the first genetic study on IHPS conducted in Chinese.

**Methods:** *nNOS* -84G>A genotypes were analyzed in 56 patients and 86 controls by polymerase chain reaction and DNA sequencing. Logistic regression was used to compute odds ratios.

**Results:** Our study could not corroborate the association previously reported. While the frequency of the IHPS-associated allele (-84A) in controls (0.205) was similar to that reported for Caucasians controls, there was a dramatic difference in -84A frequencies between Caucasian and Chinese patients (0.198). Similarly, there was no difference in the *nNOS* -84G>A genotype distribution between patients and controls.

**Conclusions:** Failure to replicate the initial finding does not detract from its validity, as genetic effects may differ across populations. Differences across populations in linkage disequilibrium and/or allele frequencies may contribute to this lack of replication. The role *nNOS* in IHPS awaits further investigation.

**KEYWORDS:** Infantile Hypertrophic Pyloric Stenosis, *nNOS*, Polymorphism, Susceptibility

## INTRODUCTION

Infantile hypertrophic pyloric stenosis (IHPS; OMIM179010) is one of the most common forms of neonatal gastrointestinal obstruction requiring surgery. IHPS is characterized by the hypertrophy and hyperplasia of the circular muscle layer of the pylorus, leading to projectile and persistent vomiting, weight loss and dehydration.<sup>1,2</sup> Both open and laparoscopic pyloromyotomy are proved to be effective in relieving gastric-outlet blockage.<sup>3,4</sup> The estimated population incidence is 1–5 per 1,000 live births although this is a representative value, with a marked preponderance of males to females in a 4:1 ratio.<sup>5</sup> Although the etiology of IHPS is still unclear, genetic predisposition to IHPS is well established. IHPS has been reported to be associated with a variety of chromosomal abnormalities, including translocation of chromosome 8 and 17 and a partial trisomy of chromosome 9, and a number of inherited syndromes, such as Smith-Lemli-Opitz and Cornelia de Lange syndromes.<sup>6-8</sup> Approximately 10% of IHPS cases are familial. Twin studies showed a concordance rate of 25-40% in monozygotic twins. The recurrence risks to sibs are around 10% and 2% in males and females, respectively, and the risk of IHPS is nearly 17-fold higher in first-degree relatives than the general population.<sup>9</sup>

IHPS is a well-recognized multifactorial trait that results from the interaction between genetic and environmental factors.<sup>10</sup> Two approaches, linkage and association studies, have been widely used to uncover genetic susceptibility to IHPS. Linkage is based on the analysis of the genotypes of members of families that segregates the disease whereas association compares the genotypes of affected individuals with those of the general population. To date, five IHPS susceptibility loci have been identified (Table1). Capon *et al.*<sup>11</sup> analyzed a single large multiplex pedigree which included 10 affected individuals, and identified an underlying IHPS disease locus in chromosome 16p12-13. Everett *et al.*<sup>12</sup> using a SNP-based high-density linkage analysis in a cohort of 81 IHPS pedigrees, uncovered two IHPS disease loci

that mapped to chromosome 11q14-q22 and Xq23 respectively. In an extended IHPS family including 8 affected individuals, chromosome 16q24 was found to be linked with the disease.<sup>13</sup> The chromosome 12q24.2-q24.31 region, where the gene encoding the neuronal nitric oxide synthase (*nNOS*) maps, was found to be implicated in IHPS<sup>14</sup> through a linkage analysis conducted in 1996. However, it was not until 2004 that an association study taking *nNOS* as a candidate gene was carried out.

nNOS is a key enzyme in the synthesis of the nitric oxide (NO), which plays an important role in the relaxation of the pyloric smooth muscle.<sup>15</sup> There is a growing body of evidence indicating that reduced expression of nNOS is a common hallmark of IHPS.<sup>16-18</sup> It has been shown that the administration of exogenous NO donor to sphincter tissues with low NOS expression can restore the capacity of relaxation,<sup>19</sup> indicating that the reduced-expression of nNOS may act as a mechanism for the defective relaxation of pyloric muscle and formation of pyloric mass, thought to be responsible for the obstruction of upper alimentary tract. Moreover, animal studies in mice have shown that absence of *nNOS* gene causes a phenotype closely resembling that of human IHPS patients.<sup>20,21</sup> The *nNOS* gene, which can generate nine distinct first-exon transcripts (exon 1a-1i) in human gut by alternative promoter usage, is one of the most structurally diverse human genes identified<sup>22-24</sup> in terms of first-exon usage and complexity of expression patterns, which suggests the existence of a temporospatial regulation of its expression under different physiological or pathological conditions.

Recently, Saur *et al.*<sup>25</sup> found significantly decreased expression of total *nNOS* mRNA in pyloric tissues of IHPS patients when compared to that of normal controls. Furthermore, a SNP at nucleotide -84 (G>A, rs41279104) in the *nNOS* exon 1c proximal promoter seemed to account for the decreased expression observed. Cell lines carrying the -84AA genotype showed a 30% decreased *nNOS* expression and significant attenuation of NO/cGMP signal

pathway when compared with those carrying the -84GG genotype.<sup>25</sup> This functional SNP was also shown to be associated with IHPS in a relatively small case-control study, including 16 patients and 81 controls in a Caucasian population.

In view of the role played by *nNOS* in IHPS development we opted to pursue further investigation. We reasoned that the evaluation of the *nNOS* association in a population of different ethnic origin from that of the initial finding would definitively increase confidence in the finding and prompt follow-up studies and further investigation of the pathology underlying IHPS at molecular level. Thus, we attempted to reproduce the initial association found in Caucasians in a Chinese population in what is the first genetic study on IHPS ever conducted in Chinese population.

## **MATERIALS AND METHODS**

### **Patients and control samples**

This study consisted of fifty-six incident patients (46 male and 10 female) with sporadic IHPS treated with pyloromyotomy, and eighty-six healthy unrelated Han Chinese (70 male and 16 female) as controls. All patients were consecutively recruited from January 2003 to January 2009 at Shenzhen Children's Hospital. Normal controls without any congenital diseases were unselected subjects from the same region during the same period. This study was approved by the Ethics Committee of Shenzhen Children's Hospital and the institutional review board of the University of Hong Kong. At recruitment, written informed consent was obtained from all participating individuals.

### **DNA sequence analyses**

DNA was extracted from peripheral blood using a QIAamp-Blood kit (Qiagen). All patients and controls were genotyped using PCR and direct sequencing. Around 10% random

samples were tested twice and the results were concordant for all of the masked duplicate sets. The PCR primers for amplification of the *nNOS* regulatory region containing -84 G>A were 5'- AAA GGT CAG AGC CTG GGA AG (forward) and 5'- ACC CCC TCT CAG ACA GTG C (reverse), which generate a 304-bp fragment and the forward primer was used for sequencing. Amplification of this DNA fragment was accomplished under the following conditions: the 25- $\mu$ l reaction mixture consisted of ~100 ng template DNA, 0.5  $\mu$ M each primer, 0.2 mM dNTP, 2.0 mM MgCl<sub>2</sub>, and 1.0 unit of Taq DNA polymerase (Applied Biosystems, Foster City, CA). The reaction was carried out in the following conditions: an initial melting step of 5 min at 95 °C, followed by 35 cycles of 30 s at 94 °C, 30 s at 58 °C, and 45 s at 72 °C, and a final elongation step of 7 min at 72 °C. PCR products were sequenced using an ABI PRISM<sup>®</sup> Big Dye<sup>™</sup> Terminator v 3.1 Cycle sequencing kit (Applied Biosystems, Foster City, CA) and an ABI 3730 automated sequencer (Applied Biosystems, Foster City, CA).

### **Statistical analysis**

The association between the polymorphism and risk of IHPS was estimated by odds ratios (ORs) and their 95% confidence intervals (CIs), which were calculated by unconditional logistic regression. Hardy-Weinberg equilibrium (HWE) was tested by Chi square test.

### **RESULTS**

In this study, we examined a total of 56 sporadic Chinese IHPS patients and 86 ethnically and gender matched controls by direct sequencing the G>84A polymorphism in the regulatory region of *nNOS*. Table 2 shows allele frequencies and genotype distributions of *nNOS* in cases and controls. The allele frequencies for *nNOS* -84A allele were 0.205 in cases

and 0.198 in controls, thus no significantly different ( $p = 0.875$ ). Among controls, the genotype distribution was in Hardy-Weinberg equilibrium ( $p = 0.265$ ), supporting the correctness of our genotyping procedure. Also, there was not differences in the genotype distribution of *nNOS* -84G>A between cases and controls. The frequencies of *nNOS* -84GG and GA genotypes in cases were similar to those in controls (66.1% and 26.8% versus 66.3% and 27.9%,  $p = 0.922$ ), and the same was true with the frequencies of rare homozygous AA genotype (7.1% in patients versus 5.8% in controls,  $p = 0.766$ ). We also used unconditional logistic regression model to estimate the association between the genotypes and risk of IHPS. The results show there were no association between the genotypes of G-84A site at the *nNOS* gene and risk of IHPS, even when the GA and AA genotypes were combined (OR:1.01; 95% CI = 0.47-2.19).

## DISCUSSION

Numerous studies have been conducted to quest for IHPS susceptibility loci,<sup>11-14,25</sup> but thus far, apart from the reported association of the condition with *nNOS* in the Caucasian population, no other causal gene has been identified. We reasoned that the evaluation of the *nNOS* association in a population of different ethnic origin would definitively increase confidence in the finding and prompt follow-up studies and further investigation of the pathology underlying IHPS at molecular level. Nevertheless, we failed to replicate the initial *nNOS*-IHPS association.

Failure to replicate the initial finding in a different population does not detract from its validity, since there is genuine diversity of the genetic effects in different populations as exemplified by the studies<sup>26,27</sup> linking cancer to a polymorphism in the *CASP8* promoter. The *CASP8* SNP was shown to be associated with susceptibility to multiple cancers in Chinese but not in Caucasians.

Lack of replication of an association in a different population may also be due to the following related scenarios: a) linkage disequilibrium (LD) differences across populations; b) large differences in allele frequencies in which case, some true associations may not be replicated, regardless of the sample size of the study; c) false positives in the original claims.

We looked into each of these scenarios. If failure to replicate was due to differential linkage disequilibrium, that would mean that the marker under scrutiny is not causal and it cannot be used as a prognostic/diagnostic test across different populations. Using HapMap phase III data, we investigated the LD in the *nNOS* region in both Caucasians and Chinese. LD is quite similar in both populations (data not shown), as are the frequencies of the IHPS-associated allele in Caucasian and Chinese controls (0.173 and 0.198 respectively). Even if the frequency of  $-84G>A$  differed across populations, the biological impact on the risk to disease would be consistent<sup>28</sup>, and the only reason preventing the replication of the association of this variant with modest effect with IHPS, would be the sample size studied. Therefore, we believe that scenarios a) and b) can be ruled out as the reasons behind the discrepancy across populations regarding the association of the *nNOS* polymorphism with IHPS. Many gene-disease associations proposed to date have not been consistently replicated across different populations, and this reflected false positives in the original claims in many cases.

We acknowledge that the study presented here has limitations, namely small sample size, a problem inherent to low incidence complex diseases. Yet, as stated above, we believe that increasing the sample size would not have changed our results; after all, the sample sizes are comparable. Interestingly, the original association described by Saur *et al.*<sup>25</sup> could not be replicated by Chung *et al* group even in a similar study conducted on Caucasian individuals.<sup>29</sup> Indeed, the role of *nNOS* in IHPS awaits further investigation.



Recent advances indicated that a complex system, including pyloric smooth muscle, the interstitial cells of Cajal, gastrointestinal hormones and enteric nervous system, may be involved in the development of IHPS<sup>29</sup>. Given the high number of genes involved in these pathways, it is reasonable to hypothesize that IHPS may be caused by polymorphisms or mutations occurring in different genes. Therefore, it would be necessary to systematically analyze the genes involved in the relaxation of pylorus to genetically dissect IHPS.

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**Table 1:** Genetic Studies on IHPS

References	Type	Ethnicity	Sample size	IHPS susceptibility loci
Chung, <i>et al.</i> [14]	Linkage analysis	Caucasian	27 families (229 individuals; 87 with IHPS)	<i>NOS1</i> (12q12.2-q24.31)
Capon, <i>et al.</i> [11]	Linkage analysis	Caucasian	3-generation family (10 affected)	16p13-p12
Everett, <i>et al.</i> [12]	Linkage analysis	Caucasian	81 families (302 individuals; 206 with IHPS)	11q14-q22 and Xq23i
Everett, <i>et al.</i> [13]	Linkage analysis	Caucasian	1 family, (8 with IHPS)	16q24
Saur, <i>et al.</i> [25]	Association study	Caucasian	Case-control; 16 IHPS and 81 controls	-84 (G>A) of <i>nNOS</i> has been identified to be associated with IHPS



**Table 2:** *nNOS* -84 G >A genotypes in cases and controls and their association with IHPS

Genotypes	Cases (n=56)		Controls (n=86)		OR (95%CI)
	Number	(%)	Number	(%)	
GG	37	(66.1)	57	(66.3)	1.00
GA	15	(26.8)	24	(27.9)	0.96 (0.42-2.21)
AA	4	(7.1)	5	(5.8)	1.23 (0.26-5.77)
GA+AA	19	(33.9)	29	(33.7)	1.01(0.47-2.19)
A allele	0.205		0.198		<i>P</i> = 0.875