Association of IRX6 rs6499755 and HAAO rs3816183 polymorphisms with hypospadias susceptibility in Chinese population

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Research Article

Keywords: hypospadias, single nucleotide polymorphism, IRX6, HAAO, Case-Control Study, Chinese population

Posted Date: June 17th, 2022

DOI: https://doi.org/10.21203/rs.3.rs-1748536/v1

Abstract

Background

Hypospadias is one of the most common male congenital external genital malformations anomalies with unclear and multifactorial etiology. Our study is aimed to investigate whether *IRX6* rs6499755 and *HAAO* rs3816183 polymorphisms are susceptible to hypospadias in the Chinese population.

Methods

We enrolled 113 patients with hypospadias and 182 healthy controls in the case-control study. Genotyping of single nucleotide polymorphisms (SNPs) was performed using High Resolution Melting (HRM). 113 hypospadias cases were further divided into anterior, middle and posterior subgroups for analysis. Otherwise, we performed a meta-analysis to evaluate the relationship in multiple populations.

Results

The risk allele [C] of *IRX6* rs6499755 was significantly associated with susceptibility to general hypospadias (OR = 1.547, $p = 0.01$), anterior hypospadias (OR = 3.579, $p = 0.003$) and posterior hypospadias (OR = 1.737, $p = 0.005$). Besides, CC genotype carriers showed an increased risk of hypospadias compared with CT + TT carriers (OR = 1.832, $p = 0.026$). The risk allele [T] of *HAAO* rs3816183 associated with susceptibility to anterior/middle hypospadias (OR = 1.775, $p = 0.046$). A combined analysis of both two SNPs was performed, showing a cumulative effect (OR = 1.337, $p = 0.02$). The results of meta-analysis (including 3789 cases and 9241 controls) indicated that rs6499755 and rs3816183 were significantly associated with hypospadias (both p 0.00001).

Conclusions

IRX6 rs6499755and *HAAO* rs3816183 polymorphisms are associated with hypospadias in a Chinese population, and the risk alleles of both two variants cluster more frequently in patients. The meta-analysis supports the hypothesis that rs6499755 and rs3816183 are the susceptibility loci for hypospadias. Further studies are needed to make the pathogenesis clearly.

Introduction

Hypospadias is the second most common congenital anomalies in newborn males after undescended testis, and characterized by proximal displacement of abnormal urethral meatus, penile curvature, and a ventrally deficient hooded foreskin[1]. According to the preoperative meatal position, hypospadias is often classified in anterior or distal (glandular, coronal, subcoronal), middle (mid penile), and posterior or proximal (posterior penile, penoscrotal, scrotal, and perineal)[2]. The anterior/middle position is the most common (80% 85%) and considered mild, while the posterior cases are considered severe[3]. The prevalence of hypospadias is 5 50 per 10,000 live births, and showing an increasing trend in the worldwide[4]. Up to now, the etiology of hypospadias is not clear. It is generally accepted that hypospadias is a highly heterogeneous condition subject, resulting from genetic predisposition and environmental factors, including parental risk, fetal risk, ethnic background, and geographic risk etc[5,6].

Single nucleotide polymorphisms (SNPs) analysis is one of important methods to analyze genetic susceptibility of common complex diseases. In 2014, a Genome-wide association study (GWAS) is performed on a European population and identifies 17 SNPs, which were associated with hypospadias independently[7]. In 2019, all of 17 SNPs are replicated performed to a Japanese case-control study (including 169 patients and 1148 controls), and only rs6499755 of *IRX6* and rs3816183 of *HAAO* are found significantly associated with susceptibility to hypospadias[8]. In 2022, the association between *HAAO* rs3816183 polymorphisms is replicated performed again in a cohort of Southern Han Chinese population (enrolled 577 patients and 654 controls). However, unlike the results of the Japanese study, the study based on the Chinese population only suggests an association between rs3816183 polymorphism and anterior/middle hypospadias, and no evidence for risk in the entirety hypospadias[9].

It is generally accepted that the existence of significant ethnic differences in genetic factors is objective, therefore evaluate the effectiveness of each locus in different ethnic groups is necessary. This case-control study was designed to investigate whether there is any meaningful relationship between *IRX6* rs6499755 and *HAAO* rs3816183 polymorphisms with hypospadias in the Northern Han Chinese population. In addition to this, we tried to further evaluate the relationship between these two SNPs and hypospadias in multiple populations through a meta-analysis, using combined data from previous studies of different races and results of our study.

Materials And Methods

Case-control analysis

Case and control samples

We enrolled 113 patients (median age at diagnosis 0.92 years) with hypospadias and 182 healthy children (median age at diagnosis 5.0 years) as controls from Tianjin Children's hospital, a regional pediatric medical center in North China (Table S1). The diagnosis of patients was made by experienced pediatric urologists. According to the preoperative urethral meatus position, the patients were classified into three subgroups: anterior hypospadias (13 patients with glandular, coronal, or subcoronal hypospadias), middle hypospadias (20 patients with mid penile hypospadias), and posterior hypospadias (73 patients with

posterior penile, penoscrotal, scrotal, or perineal hypospadias). And 7 cases could not be classified due to previously operations at other hospitals. All controls included in our study were unrelated with hypospadias, they came to hospital just for a physical examination. Informed consent was obtained from all participants or their parents/legal guardians, and the study was approved by the Ethics Committee of Tianjin Children's Hospital (Tianjin, China)

DNA extraction and genotyping

Human genomic DNA was extracted from peripheral blood samples of all patients and controls using a Genomic blood DNA mini kit (Beijing ComWin Biotech, Beijing, China) according to the manufacturer's protocol, and stored at -20°C for later use. Quantitative measurements of DNA concentrations were obtained using a Nanodrop Spectrophotometer (Nanodrop Technologies, Thermoscientific, Wilmington, DE, USA). The website of NCBI (https://blast.ncbi.nlm.nih.gov/Blast.cgi) was used to designed primers, and Sanger sequencing was used to seek the template genotypes for each SNPs locus. The genotypes of two SNPs were determined by High Resolution Melting (HRM) technology. Finally, the samples that could not be resolved clearly by HRM were sent to Sanger sequencing for accurate results.

Statistical analysis

SPSS software (version 26.0) was used for the data analysis. Chi-square test was used to calculate genotype and allele frequencies in cases and controls. A chi-squared goodness-of-fit test was conducted to examine the consistency between the genotype frequencies of the samples and Hardy-Weinberg equilibrium (HWE). Logistic regression was performed to investigate the association between frequency of risk alleles and hypospadias risk. Odds ratios (ORs) and confidence intervals (CIs) were calculated using the non-susceptible locus as the reference. A *p*-value of 0.05 was considered statistically significant for all tests.

Meta-analysis

We searched three English databases (Web of Science, PubMed, and EMBASE) and three Chinese databases (VIP, Wanfang, and CNKI), to retrieve all pertinent association studies involving rs6499755 of *IRX6* or/and rs3816183 of *HAAO* and hypospadias risk published before April 15, 2022. The keywords were "*IRX6*", "*HAAO*", "rs6499755", "rs3816183", "single nucleotide polymorphisms", and "hypospadias". The data used for meta-analysis was combined the collected data from previous researches and the results of our case-control study. Review Manager 5.4 software[10] was used for the meta-analysis. The heterogeneity test was undertaken by the Q test and I^2 test. If a P-value > 0.1 (Q test) as well as I^2 < 50% (I^2 test), heterogeneity was considered to be meaningless. In this study, we used Mantel-Haenszel statistical method and random effect model to calculate the Odds Ratio for all groups, and $p < 0.05$ suggested a significant difference.

Results

Results of case-control analysis

Association of IRX6 rs6499755 and HAAO rs3816183 polymorphisms with hypospadias susceptibility

A total of 113 patients and 182 controls were enrolled in this case-controls. The genotypes frequencies of controls and patients were shown in Table 1. The frequency distribution of the *IRX6* rs6499755 and *HAAO* rs3816183 genotype in the case and control groups was consistent with Hardy–Weinberg equilibrium (P > 0.05). Compared with TT genotype, CC phenotype of *IRX6* rs6499755 was associated with the increased risk of hypospadias (CC vs. TT: OR = 2.234, 95% CI = 1.173–4.254, $p = 0.014$). At the same time, the results showed that the *IRX6* rs6499755 [C] polymorphism may be associated with hypospadias susceptibility in recessive models (CC vs. CT + TT: OR = 1.832, 95% CI = 1.072–3.130, $p = 0.026$). However, there were no statistically significant associated with hypospadias risk observed in *HAAO* rs3816183 [C] polymorphism (all P > 0.05).

Table 1
Association between IRX6 rs6499755 T > C / HAAO rs3816183 T > C polymorphism and hypospadias susceptibility

Genotype	Cases (n = 113)	Controls (n = 182)	OR (95% CI)	*P*	*P* for HWE
IRX6 rs6499755					0.26
TT	27(23.89)	62(34.07)	Ref	1	
CT	50(44.25)	83(45.60)	1.383(0.781–2.451)	0.266	
CC	36(31.86)	37(20.33)	2.234(1.173–4.254)	**0.014**	
Model					
Dominant model (CC + CT vs TT)	86/27	120/62	1.646(0.969–2.796)	0.640	
Recessive model (CC vs CT + TT)	36/77	37/145	1.832(1.072–3.130)	**0.026**	
HAAO rs3816183					0.55
CC	65(57.52)	113(62.09)	Ref	1	
CT	40(35.40)	58(31.87)	1.199(0.723–1.987)	0.482	
TT	8(7.08)	11(6.04)	1.264(0.484–3.304)	0.632	
Model					
Dominant model (TT + CT vs CC)	48/65	69/113	1.209(0.750–1.951)	0.436	
Recessive model (TT vs CT + CC)	8/105	11/171	1.184(0.461–3.040)	0.725	

Values are shown as number (%); OR, odds ratio; CI, confidence interval; HWE, Hardy–Weinberg equilibrium; Ref, reference; *P*, *p*-values and significant p values (< 0.05) are in bold.

In order to further explore the possibility of a synergistic effect existence between the two SNPS loci, we calculated the number of risk alleles for each sample, and performed a combined analysis. The result revealed a cumulative effect: when the frequency of risk alleles increased, the risk of hypospadias increased (OR = 1.337, 95% CI = 1.047–1.709, p = 0.02) (Table 2).

Table 2
Distribution of the number of minor (risk) alleles in hypospadias *and controls*

Number of Minor (Risk) Alleles	Cases, n (%)	Controls, n (%)	OR (95% CI)	*P*
0	14	43	1.337(1.047–1.709)	**0.02**
1	38	65		
2	47	51		
3	10	22		
4	4	1		

OR, odds ratio; CI, confidence interval; *P*, *p*-values and significant p values (< 0.05) are in bold.

Subgroups analysis of SNPs and hypospadias risk according to clinical classifications

A total of 106 patients were classified into three subgroups (anterior, middle and posterior) based on the preoperative urethral meatus position. The risk allele [C] of *IRX6* rs6499755 was associated with an increased susceptibility to overall hypospadias (OR = 1.547, 95% CI = 1.108–2.160, p = 0.01), anterior (OR = 3.579, 95% CI = 1.468–8.724, p = 0.003) and posterior hypospadias (OR = 1.737, 95% CI = 1.179–2.560, p = 0.005). The risk allele [T] of *HAAO* rs3816183 had an associated with increased risk of middle (OR = 2.130, 95% CI = 1.072–4.232, p = 0.028) and anterior/middle (OR = 1.775, 95% CI = 1.005–3.135, p = 0.046) hypospadias. Nevertheless, no significant association was found in other subgroups (all P > 0.05) (Table 3).

Table 3
Association of *IRX6* rs6499755 and *HAAO* rs3816183 with different groups of hypospadias in Chinese population

SNPs	Nearby gene	Minor allele	MAF		Anterior only		Anterior + Middle		Middle only		Posterior only		All Cases	
			Cases	Controls	OR (95%CI)	P	OR (95%CI)	P	OR (95%CI)	P	OR (95%CI)	P	OR (95%CI)	P
rs6499755	IRX6	C	0.540	0.431	3.579 (1.468–8.724)	**0.003**	1.099 (0.649–1.861)	0.726	0.500 (0.242–1.032)	0.057	1.737 (1.179–2.560)	**0.005**	1.547 (1.108–2.160)	**0.01**
rs3816183	HAAO	T	0.248	0.220	1.308 (0.531–3.211)	0.558	1.775 (1.005–3.135)	**0.046**	2.130 (1.072–4.232)	**0.028**	0.996 (0.627–1.585)	0.988	1.169 (0.791–1.729)	0.432

MAF, minor (risk) allele frequency; OR, odds ratio; CI, confidence interval; SNPs, single nucleotide polymorphisms; *P*, *p*-values and significant *p* values (< 0.05) are in bold

Results of meta-analysis

Three relevant articles were obtained by searching six databases, including 3676 cases and 9059 controls[7–9]. We subsequently conducted a meta-analysis on two SNPs loci by combining the data of this study and the included researches. We calculated the ORs and 95% CI of the risk alleles according to the different races involved in the four studies.

Three included studies assessed the association between the risk alleles [C] of *IRX6* rs6499755 and the risk of hypospadias. The results showed a statistically significant increased risk of hypospadias (ORs = 1.46, 95% CI: 1.28–1.66, $p < 0.00001$) (Fig. 1-A). The heterogeneity ($p = 0.01$ and $I^2 = 66\%$) was considered to be moderate. There might be a mild related between the *IRX6* rs6499755 risk alleles [C] and anterior/middle hypospadias risk (ORs = 1.30, 95% CI: 0.99–1.70, $p = 0.06$) (Fig. 1-B).

Four included studies investigated the association between the risk alleles [T] of *HAAO* rs3816183 and susceptibility to hypospadias, indicated a significantly increased risk of hypospadias (ORs = 1.23, 95% CI: 1.16–1.31, $p < 0.00001$) (Fig. 2-A). The heterogeneity (P = 0.59 and $I^2 = 0\%$) was considered to be meaningless. Otherwise, we noted a significantly increased risk of anterior/middle hypospadias associated with *HAAO* rs3816183 [T] (ORs = 1.39, 95% CI: 1.17–1.65, $p = 0.0002$) (Fig. 2-B). However no significantly increased risk of posterior hypospadias was found linked to *IRX6* rs6499755 [C] and *HAAO* rs3816183 [T] (all $p > 0.05$) (Figure S1 and Figure S2).

Discussion

Hypospadias is a common congenital malformation, caused by failure of fusion of the urethral folds, endodermal differentiation, and ectodermal ingrowth in gestational 8 to 20 weeks, and the severity of hypospadias depends on the time of fusion fails in embryonic period[11,12]. The etiology of hypospadias is multifactorial and highly heterogeneous, including genetic factors and environmental factors. Environmental factors include early placental malfunction, low birthweight, maternal hypertension, pre-eclampsia, maternal intrauterine diethylstilbestrol exposure, use of intracytoplasmic sperm injection (ICSI), prolonged time-to-pregnancy, high maternal BMI, primiparity, multiple pregnancy, pre-existing maternal diabetes, maternal medication use: anti-epileptic drugs etc[13–15]. It is generally believed that hypospadias is caused by multiple genetic factors rather than a single gene[16]. In order to find more suspected risk loci for hypospadias, GWAS and analysis of SNPs are increasingly used over recent years. In 2014, Geller et al[7] identified 17 SNPs in European population. Later, Kojima et al[8] replicated all of these SNPs in Japanese population found out two SNPs (rs6499755 and rs3816183) associated with hypospadias. Recently, Liu et al[9] replicated rs3816183 in Southern Han Chinese population, it was also found that there was a certain correlation between the locus and hypospadias. Our study aimed to replicate the results of rs6499755 and rs3816183 using different races. Firstly, we designed a case-control analysis to assess the relationship between these two SNPs and hypospadias susceptibility in a Northern Han Chinese population. Next, we performed a meta-analysis to evaluate the relationship in multiple races, based on the results of our study and three previous studies.

Through case-control analysis, we found that *IRX6* rs6499755 was linked to increased risk of hypospadias (OR = 1.547, $p = 0.01$), especially anterior (OR = 3.579, $p = 0.003$) and posterior hypospadias (OR = 1.737, $p = 0.005$), which probably through recessive models (OR = 1.832, $p = 0.026$). We also observed *HAAO* rs3816183 had an associated with increased risk of anterior/middle (OR = 1.775, $p = 0.046$) hypospadias, especially middle hypospadias (OR = 2.130, $p = 0.028$). Besides, there was a cumulative effect between the frequency of two risk alleles and the risk of hypospadias (OR = 1.337, $p = 0.02$). By performing meta-analysis, we found a significant associated between *IRX6* rs6499755 / *HAAO* rs3816183 with the increased risk of hypospadias (ORs = 1.46, $p < 0.00001$; ORs = 1.23, $p < 0.00001$, independently). In the subgroup of anterior/middle hypospadias, we observed a mild related with *IRX6* rs6499755(ORs = 1.30, $p = 0.06$) and a significantly associated with *HAAO* rs3816183 (ORs = 1.39, $p = 0.0002$). Nevertheless, the further biological mechanisms of *IRX6* rs6499755/ *HAAO* rs3816183 result in the increased hypospadias risks remain unclear.

IRX6 (iroquois homeobox 6) belongs to homeobox genes of the *Irx* family *IrxB* cluster[17]. The *Irx* family, as regulators of development, plays an important role in embryo proliferation and differentiation, including proliferation of early embryonic cells, directed differentiation of embryonic cells, and organ formation[18,19]. There has been reported that *Irx6* expression was observed in multiple tissues in the process of embryonic development[20,21]. During external genitalia development of male mouse embryos, *IRX6* expresses in ectodermal epithelium, particularly in dorsal ectodermal, and no expression was found in the ventral ectoderm adjacent to the urethral plate[8]. In both case-controls analysis and meta-analysis, the results revealed that the risk allele [C] of *IRX6*

rs6499755[C] increase the risk of hypospadias, which greatly increases the reliability of this kind of relationship. It is reported that SNP loci located in other homologous gene box families (such as *HOXA* cluster, *IRX3, IRX5*, *ZFHX3*, etc.) also increase the risk of hypospadias[7], which may suggest the focus of the future research. Different from the results of Kojima in Japanese population, we found the association between *IRX6* rs6499755 and increased risk of general, anterior and posterior hypospadias, and no significant association with anterior/middle hypospadias in Chinese population. However, the meta-analysis involving data from these two studies found a mild related between the SNPs and anterior/middle hypospadias risk. This may be resulted from ethnic differences, considered the high similarity of origin between the two races, it is more likely to be caused by the small sample size and the low weight of this study in the meta-analysis. In future, studies with larger sample sizes and higher quality are needed.

HAAO (3-hydroxyanthranilate-3,4-dioxygenase) is an enzyme and widely distributed in various organs. The function of HAAO is catalyzing the kynurenine pathway from tryptophan to quinolinic acid[22]. Hence, it plays a role in disorders associated with altered tissue levels of quinolinic acid, such as neuronal damage[23]. Although HAAO has been associated with fetal malformation and death in mouse models[24], there is no significant upregulation of HAAO expression was observed in the developing male mouse embryos external genitalia[8]. Up to now, there is still no evidence that HAAO is involved in human male genital development in embryonic period. In our study we found HAAO rs3816183 associated with hypospadias in both of European and Asian population, however, we lacked the data of clinical subgroup analysis in European. In Asian population, we observed the association between *HAAO* rs3816183 with anterior/middle hypospadias by subgroup analysis. Base on existing data, we doubted HAAO rs3816183 polymorphism may give rise to hinder the process of fusion of the urethral folds with the midline on the ventrum of the penile shaft, especially in middle and advanced stage by changing circulating metabolites in the kynurenine pathway in Asians[25].

Although we observed relatively positive results though our work, there are still some limitations, which remain to be improving in the future. First, the samples of the case-controls analysis are relatively small, which may result in the decrease of results veracity. A larger multicenter study is needed to determine the association between SNPs and hypospadias in Chinese population. What's more, there are few reports on the association of these two loci with hypospadias, as well as the possible existence of unpublished negative results, causing the limitation of meta-analysis. We included only European and Asian populations with unequal distribution of data weights across populations, which may influence the results. It suggests the importance of multinational collaborative to investigate the association between SNPs and hypospadias.

Overall, our study indicates that there are association between variations of *IRX6* and *HAAO* and hypospadias risk in a Chinese population. Furthermore, the results of meta-analysis support identified a strong relationship between the *IRX6* and *HAAO* polymorphisms and hypospadias susceptibility in multiple populations. Further researches with larger sample size involving multiethnic will be needed to validate our results. Besides, more in-depth and comprehensive studies are needed to explore the biological pathogenic mechanism of *IRX6* and *HAAO* leading to hypospadias.

Declarations

Ethics approval and consent to participate:

Informed consent was obtained from all participants or their parents/legal guardians, and the study was approved by the Ethics Committee of Tianjin Children's Hospital (Tianjin, China).

Availability of data and materials:

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Author Contributions Statement

Yuping Yu: Conceptualization, Writing - Original Draft, Writing - Review & Editing, Formal analysis. Nan Liu: Writing - Original Draft, Writing - Review & Editing, Investigation. Ziying Chen: Methodology, Formal analysis, Investigation, Writing - Review & Editing. Jianbo Shu: Resources, Investigation, Methodology, Writing - Review & Editing. Xiaofang Chen: Investigation, Writing - Review & Editing, Methodology. Guodong Xu: Resources, Investigation, Methodology, Writing - Review & Editing. Chunquan Cai: Conceptualization, Project administration, Funding acquisition, Writing - Review & Editing, Formal analysis, Investigation, Supervision. All authors approve the final version and they assume responsibility related to its accuracy and integrity.

Funding Source:

The present study was supported by the Natural Science Foundation of Tianjin [grant number 21JCZDJC00390], the Public Health and Technology project of Tianjin [grant number ZC20120] and the Public Health and Technology project of Tianjin [grant number ZC20063]. The sources of support had no influence on the content of the manuscript.

Disclosure of Interest

All authors declare they have no conflicts of interest.

Acknowledgements

Thanks for the supporting of "Tianjin Medical Key Discipline (Specialty) Construction Project".

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Figures

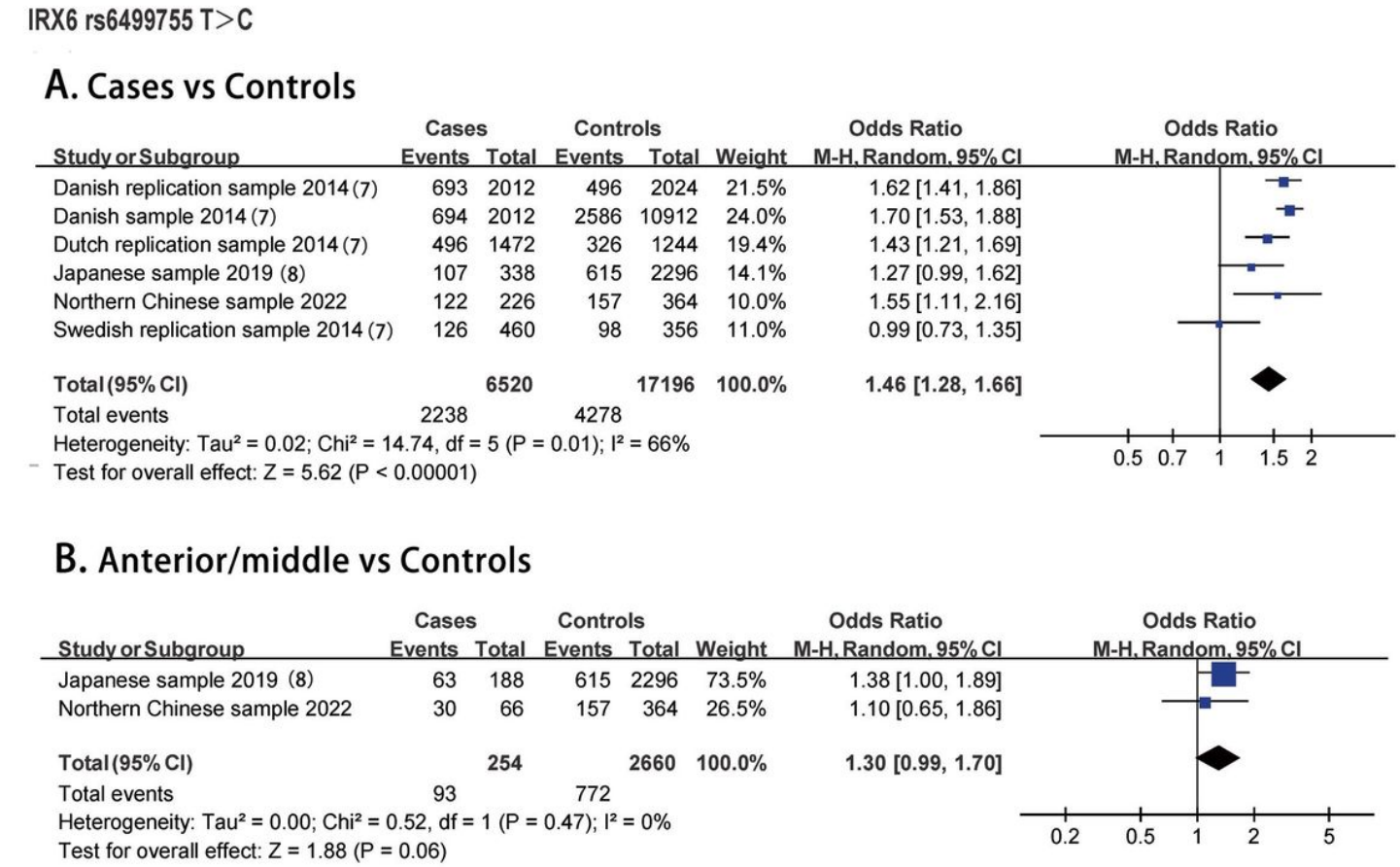

Figure 1

Forest plot of the association between *IRX6* rs6499755 and hypospadias. (A) Forest plot of the association between rs6499755 and general hypospadias. (B) Forest plot of the association between rs6499755 and general mild/moderate hypospadias.

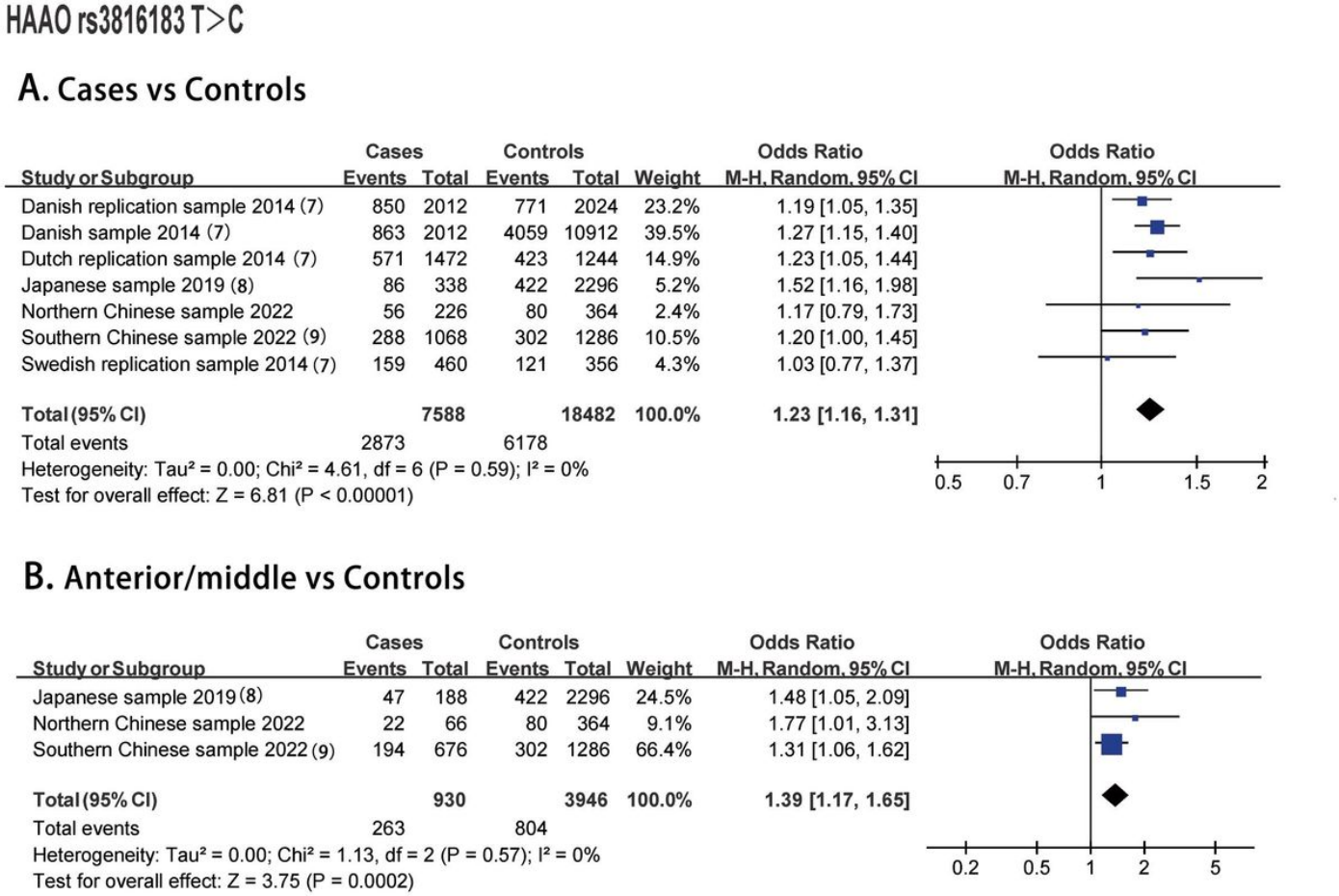

Figure 2

Forest plot of the association between *HAAO* rs3816183 and hypospadias. (A) Forest plot of the association between rs3816183 and general hypospadias. (B) Forest plot of the association between rs3816183 and general mild/moderate hypospadias.

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- Supplementaryfiles.zip