Interaction between CYP1A1/CYP17A1 polymorphisms and parental risk factors in the risk of hypospadias in a Chinese population

Yaping Mao¹,², Kang Zhang¹, Lin Ma², Xiaoyun Yun², Fengrong Ou³, Ge Liu⁴, Yi Yang⁴, Yumin Zhang¹, Xiucong Pei¹, Zhiwen Duan¹ & Mingyue Ma¹

Hypospadias (HS) is a common congenital malformation of the genitourinary tract in males and its etiology is viewed as multifactorial, and studies about gene-environment interaction in the etiology of HS are rare. A total of 152 cases and 151 controls were selected in the present study. Information before and during pregnancy from questionnaires finished by mothers of subjects were extracted, and the relating data were analyzed to determine the risk factors of HS. Meanwhile, maternal genomic DNA was genotyped for the single nucleotide polymorphisms (SNPs) of CYP1A1 rs1048943 and CYP17A1 rs4919686. Results of multivariable logistic regression analyses showed that several factors were associated with hypospadias risk. Analysis of the distributions of SNPs in CYP1A1 and CYP17A1 genes showed that the mutant genotype CC (OR = 4.87) of CYP1A1 rs1048943, and mutant genotype CC (OR = 5.82), recessive genotype AC + CC (OR = 2.17) and allele C (OR = 1.77) of CYP17A1 rs4919686 significantly increased the risk of HS. In addition, the additive gene-environment interactions were also found in several models. Several maternal risk factors that are associated with HS risk can interact with CYP1A1/CYP17A1 polymorphisms, which lead to infants vulnerable to occurrence of HS in Chinese populations.

Hypospadias (HS), one of the most common congenital reproductive malformations, is a urethral opening on the ventral surface of the penis or on the scrotum or the perineum due to incomplete fusion of the urethral folds. With the seriousness of environmental pollution intensifying in different countries, a number of related-studies have reported an increasing trend in prevalence of HS over time. While in some sites, Europe and Australia, incidence rates of HS was stable comparatively. For China, the overall prevalence of HS is 7.64 per 10 000 infants, and the eastern region was higher than the western.

The etiology of HS has proven to be multifactorial, involving genetic, endocrine and environmental factors. Furthermore, Marrocco et al. showed that the interaction between genotype and environmental factors mainly increased the risk of HS, and it might not be induced by genetic or environmental single factors. However, most studies only focused on a single type of risk factor for HS, environmental risk factor such as consumed alcohol, drugs intake during pregnancy, proximity to agricultural pesticide, maternal age (>35), and hypertension during pregnancy, or genetic risk factors such as single nucleotide polymorphism in genes related to metabolism of endogenous estrogens and normal urethral development. The importance of gene-environment interaction in the etiology of HS was only investigated in few studies, and the related genes include steroid 5 alpha-reductase (SRD5A2), activating transcription factor 3 (ATF3) and methylenetetrahydrofolate reductase.

¹Department of Toxicology, School of Public Health, Shenyang Medical College, Shenyang, Liaoning Province, 110034, China. ²Editorial Department of Journal of Shenyang Medical College, Shenyang, Liaoning Province, 110034, China. ³Department of Clinical Nutrition, The First Affiliated Hospital of China Medical University, Shenyang, Liaoning Province, 110001, China. ⁴Department of Pediatric Surgery, Shengjing Hospital of China Medical University, Shenyang, Liaoning Province, 110004, China. Yaping Mao and Kang Zhang contributed equally. Correspondence and requests for materials should be addressed to M.M. (email: mymacmu@163.com)
One possible explanation for this disease is environmental contamination. As many pollutants are metabolized by detoxifying enzymes, also polymorphisms of their encoding genes, which are able to interfere with their catalytic efficiency, may play a noteworthy role in the urogenital tissue formation. The cytochrome P450 (CYP) enzymes can metabolize various exogenous substances such as environmental chemicals and pollutants, and two enzymes of this superfamily, the aryl hydrocarbon hydroxylase (CYP1A1) and the bifunctional 17α-hydroxylase/17, 20-lyase (CYP17A1), play major roles in this metabolism. CYP1A1 is located on chromosome 15q22-q24, and is a well-studied phase I enzyme. CYP1A1 can metabolically transform not only environmental chemicals but also endogenous estrogens into more hydrophilic compounds. The T/C mutation in exon 7 of CYP1A1 (rs1048943), which is induced by some environmental chemicals such as TCDD (2,3,7,8-tetrachlorodibenzo-p-dioxin) and benzo[a]pyrene (BaP), leads to an amino acid substitution from isoleucine (Ile) to valine (Val) at codon 462. CYP1A1 I462V polymorphism shows the effect of enhancing catalytic activity. Nebert et al. reported that CYP1A1 was involved in the clearance and bioactivation of endogenous steroids including estradiol, progesterone and pregnenolone, which are necessary for the normal development of genital gland. Because normal urethral development depends on the delicate balance of these endogenous hormones, metabolites of estrogens or other hormones formed by CYP1A1 might play roles in the development of HS.

CYP17A1 is another key enzyme in steroid hormone biosynthesis pathway. The enzyme has both 17α-hydroxylase activity and 17, 20-lyase activity. CYP17A1 gene is located on chromosome 10q24.3, and is mostly expressed in adrenals and gonads, as well as plays an important role in normal physiological events such as adrenarche and puberty. CYP17A1 is required for androgen and oestrogen synthesis. Given that androgens serve as precursors to estrogens, normal estrogen signaling is also dependent on CYP17A1, which suggested that it could regulate the normal development of the fetus.

Materials and Methods

Study Population. Cases (152) were live born children diagnosed with HS at the Department of Pediatric Urology of Shengjing Hospital of China Medical University between August 2016 and September 2017. Controls (151) were randomly selected from the same hospital during the same period. Oral epithelial cells or saliva were collected from mothers of the subjects. Mothers were also invited to fulfill questionnaires concerning demographics, family history, health and lifestyle before and during pregnancy.

Case selection criteria was with isolated HS according to the International Classification of Diseases (ICD)-11. The results about whether newborns had any congenital malformations were got by obstetricians and the congenital reproductive malformations were further diagnosed by urologists. All participants were native Chinese. Cases and controls with a syndrome or chromosome abnormality and cases with a known cause of hypospadias were excluded. The specific exclusion criteria were true hermaphroditism, chordee alone, and adrenogenital syndrome. Meanwhile, to ensure independent analyses, we selected only one case or control per-family in the study after excluding the youngest brother or one of the twin brothers randomly.

DNA extraction and genotyping. We collected the oral epithelial cells or saliva from each subject’s mother by using throat swab to scrub two side of oral cavity after gargling with rinsing twice. Genomic DNA was extracted by using EZ1 DNA Investigator Kit (QIAGEN, Germany) and was stored at −80 °C immediately for further genotype analysis.

DNA samples were genotyped for the single nucleotide polymorphisms (SNPs) of CYP1A1 rs1048943 (MAF = 0.13) and CYP17A1 rs4919686 (MAF = 0.11) using the 5’-nucleotide SNP Genotyping Assay (array ID: C_25624888_50 and C_11201596_10, Applied Biosystems, Foster City, CA). The TaqMan SNP genotyping assays were performed by Applied Biosystems 7500 Fast Real-Time PCR System, which were carried out in 96-well plates in a 5 μl reaction volume containing 10 ng genomic DNA, 0.125 μl assay mix (20 ×), 2.5 μl Taqman universal PCR mastermix (2 ×), and 1.375 μl milli-Q. Cycling conditions were consisted of a pre-PCR read at 60 °C for 60 sec, then pre-denaturation step at 95 °C for 10 min, followed by 40 cycles of denaturation at 95 °C for 15 sec and annealing and extension at 60 °C for 60 sec, ended by post-PCR read at 60 °C for 60 sec. FAM (480/520 nm) and VIC (530/560 nm) fluorescence levels of PCR products were measured at 60 °C for 1 min. Genotyping was carried out in the Key Laboratory of Environmental Pollution and Microecology of Liaoning Province for quality control. After PCR, samples were genotyped using allelic discrimination in an Applied Biosystems 7500 Fast Real-Time PCR System.

Questionnaire data. After signing informed consent, parents were interviewed one-to-one with the pre-designed questionnaires by interviewer who was pre-trained by graduate advisor on the content of the questionnaire. Questionnaires contained a variety of questions about lifestyle, environmental exposure and health status before and during pregnancy, and these information were analyzed to determine the individual and environmental risk factors for HS. Infant characteristics included gender, year of birth, birth weight, premature delivery or not. Parental characteristics were family residence when mother got pregnant (rural/urban), educational level (junior high school education, senior high school education, undergraduate education, postgraduate education),
history of smoking and drinking. Besides, maternal characteristics also included age at delivery (<25, 25–29, 30–34, or ≥35 years old), drug intake before and during pregnancy (such as antibiotics, anti-abortion drugs, antiepileptic drugs, contraceptive drugs, hypnotics, anti-constipation drugs, and cough and cold medications), progesterone intake, pesticides exposure, pre-pregnancy body mass index (BMI) (<18.5, 18.5–24.9, 25.0–29.9, and ≥30.0 kg/m²), irregular menstruation (during 12 months before pregnancy), occurrence of abnormalities during pregnancy (gestational diabetes, amniotic fluid anomaly, toxemia of pregnancy, premature labor, influenza and others), diseases during pregnancy (gynecological disease, bronchial asthma, diabetes, hypertension, liver disease, heart disease, cancer, thyroid disease, epilepsy, and others).

**Statistical analysis.** The CYP1A1 rs1048943 and CYP17A1 rs4919686 genotypes frequencies in controls were tested by Hardy-Weinberg equilibrium (HWE). The crude and adjusted odds ratios (ORs) with 95% confidence intervals (95% CIs) for the independent associations of HS with parental risk factors before and during pregnancy and maternal CYP1A1 rs1048943/ CYP17A1 rs4919686 genotypes were calculated by univariable and multivariable logistic regression analyses. Meanwhile, the role of interactions between maternal risk factors and CYP1A1 rs1048943/CYP17A1 rs4919686 polymorphisms in the etiology of HS were examined as well. Relative excess risk of interaction (RERI) and attributable proportion due to interaction (AP) were calculated to assess interaction on an additive scale using the method proposed by Rothman and Excel spreadsheet developed by Andersson et al.**6**

In order to get precise results of the present study, we considered maternal age at delivery, maternal residence during pregnancy as potential confounding factors in this analysis. And the risk estimation was adjusted by these confounding factors in both univariable and multivariable logistic regression analyses. SPSS 22.0 software for Windows (IBM SPSS, Chicago, IL, USA) was used to perform statistical analyses.

**Statement of methods.** Our study was approved by the Ethics Committee of Shenyang Medical College and written informed consent was obtained from each participant. All researches were performed in accordance with relevant guidelines/regulations.

**Results**

**Study population.** The participation rates were 85% for cases and 75% for controls, resulting in a total of 163 cases and 159 controls that were available for this study. To ensure the accuracy of the investigation, we excluded 11 cases for chromosome abnormality or a known cause of HS and 8 controls for disease (such as cryptorchidism) might induced by the same cause of HS. After the exclusion step, 152 cases and 151 controls were available for risk factors analyses, while because of the failed DNA extraction of several subjects (<10%), only 148 DNA samples of cases and 147 DNA samples of controls were obtained to perform SNPs genotyping. As shown in Table 1, compared with the mothers of controls, mothers of cases were older at time of delivery (28.22 vs 27.54), had a higher proportion of living in rural areas (36.2% vs 29.8%), had a lower educational level (junior and senior high: 61.8% vs 81.2%), and also had a higher proportion of abnormal BMI (28.1% vs 21.2%).

**Association of parental factors with hypospadias.** The results of univariable logistic regression analyses showed that there were many significant differences between cases and controls, as showed in Table 2. Infants with low birth weight (LBW) (OR = 6.40) and premature delivery (OR = 3.58) were associated with the increased risk of HS. Parents with higher education could decrease the risk of HS (fathers with undergraduate level: OR = 0.39, 95%CI: 0.20–0.78, P = 0.01; fathers with postgraduate level: OR = 0.36, 95%CI: 0.17–1.76, P = 0.01; mothers with postgraduate level: OR = 0.36, 95%CI: 0.17–0.79, P = 0.01). Mothers with irregular menstruation, drug intake (including antibiotics, contraceptive drugs, and cold medications), cigarette smoking (≥1 cigarettes/day), occupational passive smoking exposure and underweight BMI (<18.5 kg/m²) before conception contributed to a higher risk of HS in offspring. Similarly, maternal factors during pregnancy such as illness (including gynecological disease, bronchial asthma, urethritis, anemia, diabetes, hypertension and liver disease), occurrence of abnormalities, progesterone intake, and occupational passive smoking exposure were also associated with the increased risk of HS. The results also showed that household monthly income was associated with HS.

Thereafter, we performed multivariable logistic regression analyses based on these variables (Table 3). The results indicated that the LBW of infants could increase the risk of HS (OR = 4.14, 95%CI: 1.58–10.87, P = 0.004). Maternal factors including irregular menstruation (OR = 3.31), passive smoking (seldom: OR = 4.02; frequently: OR = 8.75), underweight BMI (OR = 2.72) before conception, and occurrence of abnormalities (OR = 2.17) during pregnancy significantly increased the risk of HS. However, we also found that compared with no exposure to passive smoking, seldom exposure to passive smoking during pregnancy was associated with reduced risk of HS and the OR was 0.20 (95%CI: 0.06–0.66). The results also showed that higher education level was a protective factor for HS (fathers with undergraduate level: OR = 0.18, 95%CI: 0.04–0.75, P = 0.02), and lower education level was a risk factor (mothers with senior high level: OR = 3.90, 95%CI: 1.22–12.45, P = 0.02). The decreased risk of HS was also associated with household monthly income (1000–3000: OR = 0.22, 95%CI: 0.07–0.71; ≥3001: OR = 0.27, 95%CI: 0.08–0.87; 5001–10000: OR = 0.15, 95%CI: 0.04–0.52).

**Association between CYP1A1/CYP17A1 genotypes and hypospadias risk.** Genotype frequencies of CYP1A1 rs1048943 and CYP17A1 rs4919686 polymorphisms were in HWE (P > 0.05) and the distributions of genotypes and mutant alleles were shown in Table 4. The distribution of TT, TC and CC genotypes of CYP1A1 rs1048943 was 52.2%, 39.2% and 8.6% respectively, and the distribution of AA, AC and CC genotypes of CYP17A1 rs4919686 was 58.8%, 33.1% and 8.1% respectively. After adjusted for confounding factors, we found that the homozygous mutant genotype CC of CYP1A1 rs1048943 significantly increased the risk of HS (OR = 4.87, 95%CI: 1.25–18.94, P = 0.022). In addition, homozygous mutant genotype CC and recessive genotype.
AC + CC of CYP17A1 rs4919686 revealed a significantly higher risk of HS in contrast to the AA genotype (CC vs AA: OR = 5.82, 95% CI: 1.45–23.4, P = 0.013; AC + CC vs AA: OR = 2.17, 95% CI: 1.24–3.80, P = 0.007). Furthermore, the mutant allele C of CYP17A1 rs4919686 also showed an increased risk of HS (OR = 1.77, 95% CI: 1.17–2.66, P = 0.006).

Interactions between risk factors and CYP1A1/CYP17A1 polymorphisms. A 2 × 4 cross-tabs was adopted to detect the gene-environment interactions (Supplementary Table 1), meanwhile, "RR_{11} − RR_{00} > (RR_{10} − RR_{00}) + (RR_{01} − RR_{00})" was defined as additive interaction of gene-environment. Although both of the results of gene-environment interaction were not indicated any significant difference between the groups of case and control, the additive gene-environment interactions were found in several models (Table 5), including interactions of CYP1A1 with irregular menstruation before conception, passive smoking before conception and occurrence of abnormalities during pregnancy, as well as interactions of CYP17A1 with occurrence of abnormalities during pregnancy.

Discussion

As we have known, most chronic and complex diseases are likely caused by the interactions among environmental exposure, genetic polymorphisms, and lifestyle behaviors. HS remains to be a prevalent congenital malformation in male reproductive system, with a multifarious etiology and challenging operation plans. A total of 152 live born children diagnosed with HS, 151 cases of controls without HS in hospital during the same period, and their parents were included in this study to investigate the risk factors for HS, the roles of CYP1A1/CYP17A1 polymorphisms, and the gene-environment interaction in the etiology of HS in Chinese population.

We found several factors based on questionnaire data that were associated with HS risk including LBW of infant, parental educational level, maternal factors such as passive smoking before and during pregnancy, irregular menstruation before pregnancy, occurrence of abnormalities during pregnancy and preconception BMI, and household monthly income.

LBW of infant has already been confirmed as a risk factor for hypospadias in many studies. In this study, we found that infants with LBW had a more than 4-fold increased risk for HS. A study conducted in Western Siberia by Osadchuk et al. recently revealed that frequent drinking and smoking in young men could alter the hormonal and metabolic balance and would increase the risk of reproductive disorders ultimately. However, Carmichael et al. found that pregnant women exposed to secondhand smoke at home in early pregnancy was associated with reduced HS risk, which was similar with our investigation about seldom maternal exposure to secondhand smoke during pregnancy (OR = 0.20). Nevertheless, for exposure to passive smoking before conception in present study, significant increased trends for HS risk were observed in seldom and frequently exposure
compared to non-exposure, which might due to constitution of mothers before conception was more sensitivity and vulnerable. Irregular menstruation has been considered as a women's poor reproductive health and a marker of metabolic disorders, therefore, it might disrupt the balance of hormones in vivo. Our finding indicated that irregular menstruation before conception was a risk factor as well. Occurrence of abnormalities during pregnancy in this study, including gestational diabetes, amniotic fluid anomaly, toxaemias of pregnancy, influenza and premature delivery, was found to increase the risk, which was similar with the results of published papers. Akre et al. found that infants born to obese mothers (BMI: ≥30 kg/m²) was associated with a more than 2-fold increased risk of HS compared with infants born to mothers with a normal weight (BMI: 20–24 kg/m²). However, Adams et al. found that maternal obesity was not a cause of HS in male infants. Our results showed that HS risk was neither associated with overweight (BMI: 25–29.9 kg/m²) nor with obese (BMI: ≥30 kg/m²), but with underweight (BMI: <18.5 kg/m²) with an OR of 2.72, which was consistent with the results of Rankin.

| Risk Factors                  | Cases N (%) | Controls N (%) | OR (95%CI) | P*       |
|------------------------------|-------------|----------------|------------|----------|
| **Of Infant**                |             |                |            |          |
| Low Birth Weight             | No 102 (67.1%) | 140 (92.7%) | 1.00 (referent) |          |
|                              | Yes 50 (32.9%) | 11 (7.3%)  | 6.40 (1.90–6.74) | 0.00     |
| Premature delivery           | No 104 (69.3%) | 135 (89.4%) |            |          |
|                              | Yes 46 (30.7%) | 16 (10.6%)  | 3.58 (1.90–6.74) | 0.00     |
| **Of Father**                |             |                |            |          |
| Educational level            | Junior High 34 (22.4%) | 20 (13.2%) | 1.00 (referent) |          |
|                              | Senior High 55 (36.2%) | 35 (23.2%)  | 0.98 (0.49–1.98) | 0.96     |
|                              | Undergraduate 40 (26.3%) | 61 (40.4%)  | 0.39 (0.20–0.78) | 0.01     |
|                              | Postgraduate 23 (15.1%) | 35 (23.2%)  | 0.36 (0.17–0.76) | 0.01     |
| **Of Mother**                |             |                |            |          |
| Irregular menstruation       | No 116 (73.6%) | 138 (91.4%) | 1.00 (referent) |          |
|                              | Yes 36 (23.7%) | 13 (8.6%)   | 3.26 (1.63–6.51) | 0.001    |
| Drug intake                  | No 133 (73.4%) | 127 (84.1%) | 1.00 (referent) |          |
|                              | Yes 39 (25.7%) | 24 (15.9%)  | 1.76 (0.99–3.15) | 0.05     |
| Occurrence of illness        | No 114 (75%) | 131 (86.8%) | 1.00 (referent) |          |
|                              | Yes 38 (25%) | 20 (13.2%)  | 2.01 (1.10–3.71) | 0.02     |
| Occurrence of abnormalities  | No 97 (63.8%) | 123 (81.5%) | 1.00 (referent) |          |
|                              | Yes 55 (36.2%) | 28 (18.5%)  | 2.47 (1.44–4.22) | 0.001    |
| Progesterone intake          | No 112 (73.7%) | 128 (73.7%) | 1.00 (referent) |          |
|                              | Yes 40 (26.3%) | 23 (15.2%)  | 1.89 (1.05–3.39) | 0.03     |
| Cigarette smoking            | No 143 (94.1%) | 150 (99.3%) | 1.00 (referent) |          |
|                              | Yes 9 (5.9%)  | 1 (0.7%)    | 8.77 (1.09–70.53) | 0.04     |
| Passive smoking exposure     | No 96 (63.2%) | 95 (62.9%)  | 1.00 (referent) |          |
|                              | Seldom 34 (22.4%) | 47 (31.1%)  | 0.74 (0.43–1.27) | 0.28     |
|                              | Frequently 22 (14.5%) | 9 (6.0%)    | 2.58 (1.11–6.00) | 0.03     |
| Passive smoking exposure     | No 112 (73.7%) | 99 (65.6%)  | 1.00 (referent) |          |
|                              | Seldom 25 (16.4%) | 44 (29.1%)  | 0.51 (0.29–0.91) | 0.02     |
|                              | Frequently 15 (9.9%) | 8 (5.3%)    | 1.60 (0.64–3.99) | 0.32     |
| Pre-pregnancy BMI             | Normal 99 (71.9%) | 119 (78.8%) | 1.00 (referent) |          |
|                              | Underweight 32(21.1%) | 16(10.6%)  | 2.58 (1.31–5.09) | 0.01     |
|                              | Overweight 17(11.2%) | 15(9.9%)    | 1.18 (0.55–2.5) | 0.68     |
|                              | Obese 4(2.6%)  | 10(7%)      | 3.21 (0.34–30.60) | 0.31     |
| Educational level            | Junior High 36(23.7%) | 26(17.2%)  | 1.00 (referent) |          |
|                              | Senior High 58(38.2%) | 38(25.2%)  | 1.13 (0.59–2.18) | 0.71     |
|                              | Undergraduate 38(25.0%) | 47(31.1%)  | 0.58 (0.29–1.16) | 0.13     |
|                              | Postgraduate 20(13.2%) | 40(26.5%)  | 0.36 (0.17–0.79) | 0.01     |
| Family income per month      | <1000 22 (14.5%) | 6 (4.0%)    | 1.00 (referent) |          |
|                              | 1000–3000 43 (28.3%) | 38(25.2%)  | 0.336 (0.12–0.93) | 0.04     |
|                              | 3001–5000 46 (30.3%) | 43(28.5%)  | 0.32 (0.11–0.88) | 0.03     |
|                              | 5001–10000 33 (21.7%) | 59(39.1%)  | 0.19 (0.07–0.55) | 0.002    |

Table 2. Univariable logistic regression analyses of potential risk factors for HS. *Adjusted for maternal age at delivery and residence during pregnancy. **Irregular menstruation during 12 months before pregnancy. †Drug intake during 3 months before pregnancy. ‡During pregnancy. $Occupational passive smoking exposure ≥15 mins/day: Seldom: 1–2 days per week; Frequently: ≥3 days per week. ‡Underweight: <18.5 kg/m²; Normal: 18.5–24.9 kg/m²; Overweight: 25–29.9 kg/m²; Obese: ≥30 kg/m². ‡Unit of family income per month: RMB.
### Table 3. Multivariable logistic regression analyses of the risk factors for HS. *Adjusted for maternal age at delivery, and residence during pregnancy. aIrregular menstruation during 12 months before pregnancy. bDuring pregnancy. cOccupational passive smoking exposure ≥ 15 mins/day: Seldom: 1~2 days per week; Frequently: ≥ 3 days per week. dUnderweight: < 18.5 kg/m²; Normal: 18.5~24.9 kg/m²; Overweight: 25~29.9 kg/m²; Obese: ≥ 30 kg/m². eUnit of family income per month: RMB.

| Risk factors                  | B     | S     | Wald | OR (95%CI)     | P*   |
|-------------------------------|-------|-------|------|----------------|------|
| Of Infant                     |       |       |      |                |      |
| Low birth weight              | No    | 1.00  |      |                |      |
|                               | Yes   | 1.42  | 0.49 | 8.34           | 4.14 (1.58–10.87) | 0.004 |
| Of Father                     |       |       |      |                |      |
| Educational level             | Junior High | 1.00 (referent) | | | | |
|                               | Senior High | −0.96 | 0.61 | 2.50           | 0.38 (0.12–1.26) | 0.11 |
|                               | Undergraduate | −1.72 | 0.73 | 5.53           | 0.18 (0.04–0.75) | 0.02 |
|                               | Postgraduate | −0.96 | 0.81 | 1.40           | 0.38 (0.08–1.88) | 0.24 |
| Of Mother                     |       |       |      |                |      |
| Irregular menstruation BPa   | No    | 1.00  |      |                |      |
|                               | Yes   | 1.20  | 0.43 | 7.73           | 3.31 (1.42–7.71) | 0.005 |
| Occurrence of abnormalities DPb | No | 1.00  |      |                |      |
|                               | Yes   | 0.77  | 0.39 | 3.85           | 2.17 (1.00–4.70) | 0.05 |
| Passive smoking exposure BPc  | No    | 1.00  |      |                |      |
|                               | Seldom | 1.39  | 0.58 | 5.26           | 4.02 (1.29–12.58) | 0.02 |
|                               | Frequently | 2.17 | 0.90 | 5.82           | 8.75 (1.50–50.93) | 0.02 |
| Passive smoking exposure DPc  | No    | 1.00  |      |                |      |
|                               | Seldom | −1.60 | 0.61 | 6.97           | 0.20 (0.06–0.66) | 0.01 |
|                               | Frequently | −1.10 | 0.98 | 1.26           | 0.33 (0.05–2.27) | 0.21 |
| Educational level             | Junior High | 1.00 (referent) | | | | |
|                               | Senior High | 1.36  | 0.59 | 5.26           | 3.90 (1.22–12.45) | 0.02 |
|                               | Undergraduate | 1.07  | 0.71 | 2.25           | 2.90 (0.72–1.67) | 0.13 |
|                               | Postgraduate | 0.54  | 0.79 | 0.47           | 1.71 (0.37–8.03) | 0.50 |
| Pre-pregnancy BMIe            | No    | 1.00  |      |                |      |
|                               | Underweight | −0.10 | 0.46 | 0.05           | 0.90 (0.37–2.22) | 0.82 |
|                               | Obese   | 0.77  | 1.40 | 0.30           | 2.16 (0.14–33.79) | 0.58 |
| Family income per monthf     | <1000  | 1.00  |      |                |      |
|                               | 1000–3000 | −1.51 | 0.60 | 6.38           | 0.22 (0.07–0.71) | 0.01 |
|                               | 3001–5000 | −1.31 | 0.59 | 4.86           | 0.27 (0.08–0.87) | 0.03 |
|                               | 5001–10000 | −1.91 | 0.64 | 8.84           | 0.15 (0.04–0.52) | 0.003 |
|                               | >10000  | −0.90 | 0.90 | 1.02           | 0.41 (0.07–2.35) | 0.31 |

### Table 4. Effect of CYP1A1 rs1048943 and CYP17A1 rs4919686 polymorphisms on the risk of HS. *Adjusted for maternal age at delivery, and residence during pregnancy.

| Gene          | Case (n = 148) No. (%) | Control (n = 147) No. (%) | Crude OR | Adjusted OR | 95%CI | P* | P for HWE |
|---------------|------------------------|---------------------------|----------|-------------|------|----|----------|
| CYP1A1 rs1048943 |            |                           |          |             |      |    |          |
| TT            | 77 (52.2%)            | 81 (55.1%)                 | Ref (1.00) |             |      |    | 0.14    |
| TC            | 58 (39.2%)            | 62 (42.2%)                 | 0.94     | 0.90        | 0.52–1.57 | 0.72 |
| CC            | 13 (8.6%)             | 4 (2.7%)                   | 4.44     | 4.87        | 1.25–18.94 | 0.02 |
| TC + CC       | 71 (47.8%)            | 66 (44.9%)                 | 0.91     | 0.94        | 0.55–1.61 | 0.82 |
| T             | 212 (71.6%)           | 224 (76.2%)                | Ref (1.00) |             |      |    |          |
| C             | 84 (28.4%)            | 70 (23.8%)                 | 1.27     | 0.88–1.83   | 0.21 |
| CYP17A1 rs4919686 |          |                           |          |             |      |    | 0.93    |
| AA            | 87 (58.8%)            | 104 (70.7%)                | Ref (1.00) |             |      |    |          |
| AC            | 49 (33.1%)            | 40 (27.2%)                 | 1.406    | 1.80        | 1.01–3.20 | 1.80 |
| CC            | 12 (8.1%)             | 3 (2.1%)                   | 4.838    | 5.82        | 1.45–23.4 | 0.01 |
| AC + CC       | 61 (41.2%)            | 43 (29.3%)                 | 1.64     | 2.17        | 1.24–3.80 | 0.01 |
| A             | 223 (75.3%)           | 248 (84.4%)                | Ref (1.00) |             |      |    |          |
| C             | 73 (24.7%)            | 46 (15.6%)                 | 1.77     | 1.17–2.66   | 0.01 |
well-trained, and all statistical analysis were adjusted for confounding factors, so the results are credible. Second, reduce uncertain information, questions of most factors were relatively easy to remember, the interviewer was these data could not be verified and inaccurate data might lead to misclassification due to recall bias. In order to
table data could not be verified and inaccurate data might lead to misclassification due to recall bias. In order to
table data could not be verified and inaccurate data might lead to misclassification due to recall bias. In order to
Our study still have several limitations. First, the nature of our study is case-control study
b) During pregnancy.

| Gene     | Risk factors                  | Cases | Controls | OR (95%CI) | RERI (95%CI) | AP (95%CI) |
|----------|-------------------------------|-------|----------|------------|--------------|------------|
| CYP1A1   | Occurrence of abnormalities DP |       |          |            |              |            |
| TT No    | 39                            | 47    | Ref (1.00)| 0.31       | (2.23–2.85)  | 0.12       | (0.20–1.23) |
| TC + CC No | 54                          | 54    | 0.98 (0.53–1.80) | 2.28 (1.04–4.98) | 1.10 (0.50–2.41) | 1.04 (0.41–2.66) |
| TT Yes   | 23                            | 23    | 5.57 (1.99–18.85) | 5.77 (1.00–5.65) | 1.04 (0.41–2.66) |            |
| CYP1A1   | Occurrence of abnormalities DP |       |          |            |              |            |
| AA No    | 57                            | 81    | Ref (1.00)| 6.98       | (3.39–17.35) | 0.77       | (0.48–1.06) |
| AC + CC No | 39                          | 39    | 1.30 (0.69–2.47) | 1.74 (0.86–3.52) | 1.74 (0.86–3.52) |            |
| AA Yes   | 31                            | 23    | 1.74 (0.86–3.52) | 9.02 (2.78–29.22) |            |            |
| AC + CC Yes | 24                         | 4     |            |            |              |            |

Table 5. Effect of interaction of CYP1A1/CYP17A1 polymorphisms-environmental risk factors on the risk of HS. a) Adjusted for maternal age at delivery, educational level and residence during pregnancy. b) Before pregnancy.

The higher education level was a protective factor for HS and the lower education level was a risk factor, which might on account of the fact that parents with a high educational level would have a better job and got well-paid so that pregnant women could get better care. Meanwhile, our study also indicated that household monthly income >1000 RMB significantly reduced the risk of HS, which might due to that for women from wealthy families known how to fend for themselves as well as have a comprehensive nutritional meal.

The biosynthesis and bioactivation of endogenous sex hormones, including estrogen and androgen, are necessary for the normal development of genital gland, which are catalyzed by enzymes of CYP1A1 and CYP17A1. As far as we know, the association between maternal CYP1A1/CYP17A1 SNPs and hypospadias have not been reported in Chinese population, let alone gene-environment interaction. In this study, analysis of the distributions of SNPs in CYP1A1 gene showed that mothers with CC mutant genotype in CYP1A1 increased the risk of HS more than 4-fold. On the contrary, a study conducted in Japan population (including 31 case mothers and 64 control mothers) by Kurahashi et al. showed that mutant genotypes (TC and TC + CC) in CYP1A1 were associated with decreased risk of HS52. The different results might be attributed to the existence of population differences and the different sample size, so further study with larger sample size and different populations is needed to confirm this association. In addition, the CYP17A1 rs4919686 polymorphism was also significantly associated at both allelic and genotypic levels with risk of HS (CC: OR = 5.82; AC + CC: OR = 2.17; allele C: OR = 1.77), which is similar with the results reported by Qin et al.53. Hence, SNPs of rs1048943 in CYP1A1 and rs4919686 in CYP17A1, which are involved in the bio-synthesis of sex hormones, have major roles in normal development of male genitalia, and may result in HS by changing the activity of the corresponding enzymes.

The pathogenesis of hypospadias is viewed as multifactorial, and induced by genes polymorphisms and gene-environment interactions, hence, it is necessary to investigate the roles of interaction between CYP1A1/CYP17A1 polymorphisms and environmental risk factors. In this study, the positive additive interactions were observed in several models (Table 5). Evidence showed that mothers with recessive genotypes (TC + CC) of CYP1A1 could interact with risk factors, including irregular menstruation, passive smoking before conception and occurrence of abnormalities during pregnancy, which resulted in the increased HS risk in male offsprings. The interaction between passive smoking with CYP1A1 rs1048943 polymorphism was also associated with other diseases, such as coronary artery disease77 and hypertension78. It is noteworthy that both of the two SNPs in CYP1A1 and CYP17A1 had additive interactions with occurrence of abnormalities during pregnancy, which indicated that occurrence of abnormalities was a definite risk factor for HS.

Here, this is by far the first comprehensive report in regard to the interactions of CYP1A1/CYP17A1 polymorphisms with environmental risk factors before and during pregnancy for the risk of HS in a Chinese population. Despite all this, our study still have several limitations. First, the nature of our study is case-control study and data was collected by interviewing the parents of cases and controls retrospectively. Hence, the validity of these data could not be verified and inaccurate data might lead to misclassification due to recall bias. In order to reduce uncertain information, questions of most factors were relatively easy to remember, the interviewer was well-trained, and all statistical analysis were adjusted for confounding factors, so the results are credible. Second,
the study group was relatively small and the number of studied SNPs was limited. In addition, the association of HS phenotypes with gene polymorphisms was not investigated and our participants only include Chinese population. Therefore, an upgraded version of our investigation including larger sample size with more SNPs of different ethnic groups is required to confirm our findings, what's more, the interaction of different phenotypes with risk factors are also needed to explore.

Conclusions
Overall, this study suggests that maternal CYP1A1/CYP17A1 polymorphisms involved in metabolism and synthesis of endogenous hormones might have a significant role in the risk of development of HS in the offsprings. In addition, several environmental risk factors are associated with HS risk and can interact with CYP1A1/CYP17A1 polymorphisms in Chinese population. Using gene-environment interaction model can elucidate the etiology of HS clearly, and provide more efficient measures. Although our study as yet still have several limitations, we stress the importance of performing stratified analyses for the different phenotypes of HS in further research.

Data Availability
The data that support the findings of this study are available from [Shengjing Hospital of China Medical University and Shenyang Medical College] but restrictions apply to the availability of these data, which were used under license for the current study, and so are not publicly available. Data are however available from the authors upon reasonable request and with permission of [Shengjing Hospital of China Medical University and Shenyang Medical College].

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Author Contributions

Yaping Mao, Kang Zhang, Lin Ma, Mingyue Ma: participation in study design, collection of questionnaire data and DNA, Taqman-genotyping analysis, statistical analysis, manuscript drafting and critical discussion. Xiaoyun Yun, Fengrong Ou, Ge Liu, Yi Yang: participation in study design, collection of questionnaire data and DNA. Yumin Zhang, Xiucong Pei, Zhiwen Duan: participation in Taqman-genotyping analysis, statistical analysis, manuscript drafting and critical discussion. All authors have read and approved the final version of the manuscript, and agree with the order of presentation of the authors.

Additional Information

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