Association between diacylglycerol kinase kappa variants and hypospadias susceptibility in a Han Chinese population

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Previous genome-wide association studies have identified variants in the diacylglycerol kinase kappa (DGKK) gene associated with hypospadias in populations of European descent. However, no variants of DGKK were confirmed to be associated with hypospadias in a recent Han Chinese study population, likely due to the limited number of single-nucleotide polymorphisms (SNPs) included in the analysis. In this study, we aimed to address the inconsistent results and evaluate the association between DGKK and hypospadias in the Han Chinese population through a more comprehensive analysis of DGKK variants. We conducted association analyses for 17 SNPs in or downstream of DGKK with hypospadias among 322 cases (58 mild, 113 moderate, 128 severe, and 23 unknown) and 1008 controls. Five SNPs (rs2211122, rs4554617, rs7058226, rs7063116, and rs5915254) in DGKK were significantly associated with hypospadias (P < 0.05), with odds ratios (ORs) of 1.64–1.76. When only mild and moderate cases were compared to controls, 10 SNPs in DGKK were significant (P < 0.05), with ORs of 1.56–2.13. No significant SNP was observed when only severe cases were compared to controls. This study successfully implicated DGKK variants in hypospadias risk among a Han Chinese population, especially for mild/moderate cases. Severe forms of hypospadias are likely due to other genetic factors.

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INTRODUCTION
Hypospadias, affecting approximately 1 out of every 750 births in Europe,1 is a common congenital disease characterized by urogenital malformation, in which the position of urethral orifice is abnormal. Hypospadias are caused by incomplete urethral fusion during gestational weeks 8 to 16.2 The phenotype of hypospadias is divided into mild (glandular), moderate (midpenile), and severe (in scrotum and perineum) depending on the abnormal location of the urethral opening.2–4 The prevalence of hypospadias has increased in developed nations since the 1960s,3 and its incidence has plateaued in recent years.6–4 However, in China, the prevalence of hypospadias has shown an increasing trend, particularly in well-developed areas.9,10 Although the etiology of hypospadias is largely unknown, genetic factors have been demonstrated to play an important role in the development of hypospadias.11,12

Hypospadias is a complex disease affected by both genetic and environmental factors. The first genome-wide association study (GWAS) of hypospadias, among a European population, was reported in 2011, which included 436 Dutch cases and 494 controls.1 In the study, two single-nucleotide polymorphisms (SNPs) (rs1934179 and rs7063116) were identified in the diacylglycerol kinase kappa (DGKK) gene, which is located on the X chromosome and is strongly associated with hypospadias.1 Subsequently, a fine-mapping study was conducted in an American population to determine whether the association of DGKK with hypospadias could be replicated in a more racially diverse population in 2013.3 Results from this second study confirmed the relationship between hypospadias and the above-mentioned SNPs. Fifteen significant SNPs were also found associated with mild and moderate cases.3 In 2014, a study of the DGKK gene was performed in a Chinese population for the first time.4

Fourteen tag SNPs in the DGKK gene were tested, but all failed to show a statistically significant association with hypospadias.4 Considering the small number of tag SNPs and limited sample size in the previous Chinese study, we aimed to conduct more comprehensive research of the DGKK gene variants with all 17 previously identified SNPs in a larger case–control study. Our hypothesis was that DGKK genetic variants would be associated with hypospadias risk in a Han Chinese population.

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MATERIALS AND METHODS

Study population
This study included 322 unrelated cases and 1008 controls, all of which were Han Chinese. Patients with hypospadias were recruited from the Department of Urology at Shanghai Children’s Hospital and were diagnosed by the Department of Urology from January 2013 to January 2014. Only patients with hypospadias without other system abnormalities were included. The severity of hypospadias cases was classified into one of three categories: mild (glandular), moderate (penile), or severe (in the scrotum or perineum) according to the position of the urethral opening. Of the 322 patients ultimately enrolled, there were 58 mild cases, 113 moderate cases, 128 severe cases, and 23 cases with unknown classification. The 1008 healthy controls were recruited from the Chinese Consortium for Prostate Cancer Genetics (ChinaPCa).

Each patient was informed of the purpose of this study, and written consent was obtained from all participants or their parent/legal guardian. Ethical approval was obtained from the Shanghai Children’s Hospital in China.

DNA extraction and genotyping
Genomic DNA was extracted from peripheral blood samples using the Gentra Puregene Blood Kit (Qiagen, Dusseldorf, Germany). Polymerase chain reaction (PCR) and extension reactions were performed according to the manufacturers’ protocol. The SNP genotypes were obtained using the MassARRAY iPLEX platform (Sequenom, San Diego, USA) and the genotyping call rates of all SNPs were >97%. Duplicated and water samples were included in each 96-well plate as PCR negative controls.

Selection of tagging SNPs
A total of 17 tagging SNPs were selected based on the Han Chinese population (CHB) in HapMap data and Genome Variation Server (http://gvs.gs.washington.edu/GVS/) with the criteria of r² > 0.8 and minor allele frequency (MAF) >0.05. The SNP rs5915330 was located in CCNB3, and the rest were in DGKK. Two SNPs (rs7063116 and rs1934179) were obtained from the first GWAS study of a European population.¹

Statistical analysis
PLINK software¹⁴ was used to test the association of each SNP with risk of hypospadias. Association analyses were performed on all cases grouped together as well as separate subgroups based on phenotype severity. Mild and moderate cases were grouped together due to their phenotype similarities. The allelic OR and 95% confidence interval (CI) were calculated using logistic regression models to estimate relative risks. We performed clamp and haplotype association analyses based on PLINK software. All P values were two-sided tests, and P < 0.05 was considered statistically significant. P = 0.003 (0.05/17) was the significance threshold through the strict Bonferroni correction. The pairwise linkage disequilibrium (LD) structure (r²) value was calculated using Haplovie 4.2 software (https://www.broadinstitute.org/haplovie/haplovie).¹⁴

RESULTS

To validate whether the genetic variants of DGKK gene were associated with hypospadias risk in a Han Chinese population, we genotyped 17 tagging SNPs. Genotype information is detailed in Table 1. Sixteen SNPs are from the DGKK gene and one SNP (rs5915330) is located on the CCNB3 gene, downstream of DGKK. With all patients grouped together, 5 of the 16 DGKK SNPs (rs2211122, rs4554617, rs7058226, rs7063116, and rs5915254) were significantly associated with hypospadias, which had P < 0.05 and were associated with increased risk, with ORs ranging from 1.64 to 1.76. Another 5 SNPs (rs5915330, rs4143304, rs1934188, rs12171755, and rs1934179) in DGKK possessed marginal P values that were slightly more than 0.05. The other seven SNPs, including the variant in CCNB3, did not reach statistical significance with P < 0.05. Because of the similar results for mild and moderate cases, we merged them into one group for analysis (data not shown). Among mild/moderate cases, ten SNPs (rs4599945, rs4143304, rs1934188, rs12171755, rs1934179, rs2211122, rs4554617, rs7058226, rs7063116, and rs5915254) had P < 0.05 and their ORs were higher compared to the analysis including all cases. For severe cases, no P values reached statistical significance and ORs tended to be closer to 1.00.

To explore whether there was a high linkage disequilibrium that existed in these SNPs, we conducted a clump analysis (Table 2). The results showed that rs4143304, rs1934188, rs12171755, rs1934179, rs2211122, rs4554617, rs7058226, rs7063116, and rs5915254 were in the same block. All of them were found to be associated with hypospadias when only mild and moderate cases were compared with controls. Four out of these nine SNPs were estimated in the same haplotype block using Haplovie 4.2 software (Figure 1).

We performed haplotype analysis in a block that included five SNPs (rs2211122, rs4554617, rs7058226, rs7063116, and rs5915254), which were in high linkage disequilibrium and identified three haplotypes (Table 3). The haplotype omnibus test revealed overall significant associations between these SNPs and hypospadias (P = 0.004). Meanwhile, individual haplotype analyses were consistent with the omnibus test and both ATGGA and GGAAG haplotypes reached significant P values (P = 0.002 and 0.001, respectively). ATGGA reflected the major allele and was the most common for each SNP, while GGAAG reflected the minor allele and was the next most common for each SNP.

DISCUSSION

In the present study, we found five SNPs (rs2211122, rs4554617, rs7058226, rs7063116, and rs5915254) that were significantly associated with hypospadias, which had P < 0.05 and were associated with increased risk, with ORs ranging from 1.64 to 1.76. Another 5 SNPs (rs5915330, rs4143304, rs1934188, rs12171755, and rs1934179) in DGKK possessed marginal P values that were slightly more than 0.05. The other seven SNPs, including the variant in CCNB3, did not reach statistical significance with P < 0.05. Because of the similar results for mild and moderate cases, we merged them into one group for analysis (data not shown). Among mild/moderate cases, ten SNPs (rs4599945, rs4143304, rs1934188, rs12171755, rs1934179, rs2211122, rs4554617, rs7058226, rs7063116, and rs5915254) had P < 0.05 and their ORs were higher compared to the analysis including all cases. For severe cases, no P values reached statistical significance and ORs tended to be closer to 1.00.

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associating with hypospadias in DGKK (P < 0.05). When only mild and moderate cases were compared with controls, ten SNPs in DGKK were significantly associated with hypospadias (P < 0.05). No risk SNP was found to be associated with severe cases. Heritability of hypospadias is approximately 65%–75%, and the risk was estimated to be increased 12- to 20-fold among first-degree relatives.15–17 A previous study also demonstrated that genetic factors have a more important role in causing familial hypospadias than intrauterine environmental factors.17 GWAS analysis is a useful method in elucidating the genetic contributions of common variants. The first GWAS of hypospadias was conducted in a European population and identified two SNPs (rs1934179 and rs7063116) of DGKK, which had compelling evidence for association with hypospadias.1 These two susceptibility genetic loci and multiple other genetic loci of DGKK were further confirmed in moderate and mild cases in an independent American study population.1 However, the first study in a Han Chinese population indicated that none of these SNPs were found to have a relationship with hypospadias.2 DGKK mRNA is found to be most abundant in the testis and second in the placenta.3 DGKK gene, located on chromosome Xp11.22, encodes diacylglycerol kinase, which plays an important role in signal transduction by modulating the balance between diacylglycerol and phosphatidic acid.18 DGKK mRNA is found to be most abundant in the testis and second in the placenta.18 In addition, real-time quantitative PCR analyses showed that DGKK was expressed in the preputial skin of 10 healthy boys and 14 hypospadias cases.6 However, little is

### Table 1: Association of diacylglycerol kinase kappa single-nucleotide polymorphisms with different phenotype severities of hypospadias in a Han Chinese population

| SNP         | Location | Nearby gene | BP | Minor allele | Major allele | MAF of cases | MAF of controls | Mild + moderate cases | Severe cases | All cases |
|-------------|----------|-------------|----|--------------|--------------|--------------|-------------------|-----------------------|-------------|-----------|
| rs5915330   | Intron   | CCNB3       | G  | 0.364        | 0.310        | 1.26 (0.90–1.76) | 0.185              | 1.36 (0.92–2.0)    | 0.117      | 1.27 (0.98–1.66) | 0.073     |
| rs5915412   | Intron   | DGKK        | A  | 0.084        | 0.093        | 0.99 (0.57–1.72) | 0.962              | 0.66 (0.31–1.39)   | 0.268      | 0.90 (0.57–1.40) | 0.627     |
| rs4472655   | Intron   | DGKK        | G  | 0.084        | 0.094        | 0.98 (0.56–1.70) | 0.933              | 0.65 (0.31–1.37)   | 0.256      | 0.89 (0.57–1.39) | 0.594     |
| rs4599945   | Intron   | DGKK        | C  | 0.244        | 0.205        | 1.56 (1.08–2.24) | 0.016              | 1.04 (0.66–1.64)   | 0.876      | 1.25 (0.93–1.69) | 0.138     |
| rs5961179   | Coding   | DGKK        | T  | 0.084        | 0.094        | 0.97 (0.56–1.70) | 0.927              | 0.65 (0.31–1.37)   | 0.254      | 0.88 (0.57–1.38) | 0.588     |
| rs7883609   | Intron   | DGKK        | C  | 0.084        | 0.094        | 0.98 (0.56–1.71) | 0.947              | 0.65 (0.31–1.38)   | 0.262      | 0.89 (0.57–1.39) | 0.610     |
| rs4143304   | Coding   | DGKK        | A  | 0.309        | 0.258        | 1.66 (1.18–2.33) | 0.003              | 0.90 (0.59–1.40)   | 0.655      | 1.29 (0.98–1.69) | 0.074     |
| rs1934181   | Intron   | DGKK        | T  | 0.309        | 0.257        | 1.67 (1.19–2.34) | 0.003              | 0.91 (0.59–1.41)   | 0.676      | 1.29 (0.98–1.71) | 0.067     |
| rs6614458   | Intron   | DGKK        | G  | 0.244        | 0.258        | 0.91 (0.62–1.33) | 0.631              | 0.92 (0.60–1.42)   | 0.705      | 0.94 (0.71–1.26) | 0.700     |
| rs12171755  | Intron   | DGKK        | A  | 0.309        | 0.261        | 1.64 (1.17–2.30) | 0.004              | 0.90 (0.58–1.38)   | 0.618      | 1.27 (0.96–1.67) | 0.089     |
| rs1934179   | Intron   | DGKK        | C  | 0.309        | 0.258        | 1.66 (1.18–2.33) | 0.003              | 0.91 (0.59–1.40)   | 0.657      | 1.29 (0.98–1.70) | 0.074     |
| rs2211112   | Intron   | DGKK        | A  | 0.188        | 0.122        | 2.08 (1.39–3.10) | 0.000              | 1.04 (0.60–1.81)   | 0.893      | 1.67 (1.19–2.34) | 0.003     |
| rs4554617   | Intron   | DGKK        | T  | 0.184        | 0.121        | 2.10 (1.41–3.15) | 0.000              | 1.05 (0.60–1.84)   | 0.855      | 1.64 (1.17–2.30) | 0.004     |
| rs7058226   | Intergenic | DGKK       | A  | 0.156        | 0.097        | 2.08 (1.35–3.21) | 0.001              | 1.15 (0.63–2.08)   | 0.649      | 1.72 (1.19–2.48) | 0.004     |
| rs7063116   | Intergenic | DGKK       | G  | 0.156        | 0.095        | 2.13 (1.38–3.30) | 0.000              | 1.18 (0.66–2.13)   | 0.592      | 1.76 (1.22–2.55) | 0.002     |
| rs5915254   | Intergenic | DGKK       | A  | 0.162        | 0.103        | 1.93 (1.25–2.97) | 0.002              | 1.15 (0.65–2.04)   | 0.631      | 1.68 (1.17–2.41) | 0.004     |
| rs8899048   | Intergenic | DGKK       | G  | 0.310        | 0.300        | 1.01 (0.71–1.43) | 0.965              | 1.07 (0.71–1.60)   | 0.751      | 1.05 (0.80–1.38) | 0.716     |

1Two SNPs selected from van der Zanden et al.1 MAF: minor allele frequency; OR: odds ratio; CI: confidence interval; SNPs: single-nucleotide polymorphisms; DGKK: diacylglycerol kinase kappa; BP: base pair

### Table 2: Clump analysis based on linkage disequilibrium between diacylglycerol kinase kappa single-nucleotide polymorphisms

| SNPs  | BP     | P     | Total | SP2  |
|-------|--------|-------|-------|------|
| rs7063116 | 50235002 | 0.002 | 8     | rs4143304, rs1934188, rs12171755, rs1934179, rs2211122, rs4554617, rs7058226, rs5915254 |
| rs5915330 | 50091920 | 0.073 | 1     | rs6614458 |
| rs4599945 | 50123966 | 0.138 | 0     | None |
| rs5961179 | 50129369 | 0.589 | 3     | rs5915412, rs4472655, rs7883609 |
| rs8899048 | 50249685 | 0.716 | 0     | None |

1These are index SNPs; 1list of other SNP names in clump; SNPs: single-nucleotide polymorphisms; BP: base pair
known about the functions of DGKK, and the biological mechanisms underlying the association between DGKK genetic variants and hypospadias risk remain elusive.

There are still several limitations in our study. First, the limited sample size may not have enough power to validate the genetic variants that exert effects on hypospadias in a Han Chinese population. Furthermore, the recruitment methods of cases and controls were different, which may have caused selection bias; thus, a larger sample size is warranted to confirm the association in a Han Chinese population. Second, little is known about the biological mechanisms of DGKK, and functional studies are needed in future projects.

CONCLUSIONS
In summary, we validated the finding of the first GWAS study of hypospadias and identified several novel SNPs in a Han Chinese population. These results from our fine-mapping study indicate that genetic variants of DGKK were associated with hypospadias in mild and moderate Han Chinese cases. Our findings support that further investigations and more comprehensive studies are warranted to address the functions and biological mechanisms of DGKK.

AUTHOR CONTRIBUTIONS
HX, XLL, JX, and FC participated in conceiving and designing the study. HX, XLL, and SZ participated in drafting the manuscript; XLL, SZ, and HTC participated in statistical analyses; HX, LY, XXL, YCH, and YQL participated in administrative, technical, and material support. All authors read and approved the final manuscript.

COMPETING INTERESTS
All authors declare no competing interests.

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