**Further evidence of association of the *DGKK* gene with hypospadias**

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**Abstract**

**Purpose:** Hypospadias is a common developmental anomaly of the male external genitalia. Previous studies conducted on West European, Californian, and Han Chinese populations have suggested that polymorphic variants of the *DGKK* gene are associated with hypospadiasis. The aim was to study the possible associations between polymorphic variants of the *DGKK* gene and hypospadias using an independent sample of the Polish population. **Methods:** Ten single nucleotide polymorphisms in *DGKK*, which were reported to have an impact on the risk of hypospadias in other populations, were genotyped using high-resolution melting curve analysis in a group of 166 boys with isolated hypospadias and 285 properly matched controls. **Results:** Two *DGKK* variants rs11091748 and rs12171755 were associated with increased risk of hypospadias in the Polish population. These results were statistically significant even after applying the Bonferroni correction for multiple comparisons (p < 0.005). All the tested nucleotide variants were involved in haplotype combinations associated with hypospadias. The global p-values for haplotypes comprising of rs4143304-rs11091748, rs11091748-rs17328236, rs1934179-rs4554617, rs1934183-rs1934179-rs4554617 and rs12171755-rs1934183-rs1934179-rs4554617 were statistically significant even after permutation correction. **Conclusions:** Our study provides strong evidence of an association between *DGKK* nucleotide variants, haplotypes and hypospadias susceptibility.

Key words: *DGKK*, diacylglycerol kinase kappa, haplotypes, hypospadias, SNP

Introduction

In hypospadias the external urethral opening is positioned abnormally at the ventral part of the penis, rather than at the tip of the glans. The majority of cases are isolated, i.e. individuals are not affected by other congenital anomalies. Hypospadias is the second most common human birth defect with an incidence of 1 in 250 live male births and its pathogenesis is complex, multifactorial, and determined by genetic, endocrine, and environmental causes [1-3]. Many linkage analyses, aiming to elucidate the molecular genetic basis of hypospadias were performed in the past, but they have met with only limited success. In part, this limited success can be attributed to the complexity of the disease, as well as to the selection of not homogenous populations for investigations [4]. Recently, two genome-wide association studies based on DNA samples from West European cases [5,6], as well as two case-control studies conducted in the California population composed primarily of Hispanic and Caucasian individuals [7] and in the Han Chinese population [8], showed that common polymorphic variants of the *DGKK* gene can increase the risk of hypospadias.

The *DGKK* gene (OMIM \*300837) located on chromosome Xp11.22 encodes the diacylglycerol kinase kappa. This enzyme is involved in the down-regulation of diacylglycerol signalling since it phosphorylates diacylglycerol, converting it to phosphatidic acid [9]. Determination of the exact associations between polymorphic variants of candidate genes and hypospadias risk might provide very important insight into the cause of hypospadias [4,10,11]. Expression of *DGKK* in preputial tissue is lower in boys with the hypospadias risk allele of rs1934179 [5]. Very recently, Shen et al. [12] reported that the enzyme Dgkk appears to be a mediator during development of mouse external genitalia.

The global burden incurred from hypospadias in terms of physical morbidity, health care expenses, emotional distress, and social dysfunction is significant for affected individuals, their families, and the health care system overall [2,11,13]. Hypospadiology remains a constantly evolving discipline with plenty of discrepancies among epidemiologic studies [1,11]. Identifying the underlying aetiology of this condition is crucial for improving prevention strategies and genetic risk counselling. The primary aim of our study was to investigate the contribution of previously reported cases of polymorphic variants of the candidate *DGKK* gene to the incidence of hypospadias in a homogenous Polish population. This study is the first to represent patients with hypospadias of East European origin as part of a replicate sample to the previously described studies. The secondary aim was to test the association between common *DGKK* haplotypes and hypospadias susceptibility using different risk models.

Methods

Patients and controls

Previous studies demonstrated familial reoccurrence of hypospadias for the anterior and middle forms of those malformations but not for posterior types, displaying the importance of genetic predisposition for hypospadias [5]. Considering this apparent etiologic heterogeneity, only isolated anterior and middle cases were included in the current study. A total of 166 unrelated boys (13 months to 10 years old) presenting with isolated hypospadias and 285 unrelated boys without congenital malformations were recruited from the Institute of Mother and Child in Warsaw. Case eligibility to the study was ascertained using the detailed medical records of each patient. The ancestry contributions were estimated to be 100% of Caucasian, Polish descent in both the hypospadias cases and the control group. DNA was isolated from peripheral blood lymphocytes using the salting-out extraction procedure. The study was approved by the local Ethics Committee. Written and oral consent was obtained from the legal guardians of all the participants.

Single nucleotide polymorphism selection and genotyping

Ten single nucleotide polymorphisms (SNPs) in *DGKK* gene, previously detected to be associated with hypospadias [5-8], were evaluated in this study (Table 1).

The genotyping was carried out by high-resolution melting curve analysis (HRM) on the LightCycler 480 system (Table 2). For quality control, approximately 10% of randomly selected samples were re-genotyped. Samples that failed genotyping were not repeated and were removed from statistical calculations.

Statistical methods

For each SNP, the Hardy-Weinberg (HW) equilibrium was evaluated in both patients and controls using Chi-square (χ2) test. Statistically significant deviation from HW expectations was interpreted as p-value < 0.05. The differences in allele frequencies between cases and controls were determined using standard χ2 test. The strength of association was estimated by Odds Ratio (OR) and corresponding 95% confidence intervals (95%CIs). The Bonferroni correction was applied to account for multiple comparisons, and p-values < 0.005 (0.05 / 10 SNPs) were interpreted as statistically significant.

The haplotype-based association analysis was performed using PLINK v1.07 (http://pngu.mgh.harvard.edu/~purcell/plink/). The omnibus haplotype test (jointly estimating all haplotype effects at a given location) for sliding windows of 2 to 4 SNPs across the gene was conducted using logistic regression. Significant p-values were corrected using the 1,000-fold permutation test. The detailed haplotype analysis was conducted for SNP combinations with statistically significant Omnibus test p-values. Haplotype-specific odds ratios (ORs) were calculated and the most common haplotypes were used as the reference. Only haplotypes with frequencies ≥ 0.01 in either cases or controls were tested.

Results

First, we analyzed the *DGKK* SNPs independently. None of the tested SNPs showed evidence of deviation from Hardy-Weinberg equilibrium in neither the cases nor the controls. After correction for multiple testing, statistically significant results of increased risk for hypospadias were observed only for carriers of the *DGKK* rs11091748 and rs12171755 variants (Table 3). The OR for individuals with the rs11091748 G allele compared to A allele carriers was 1.87 (95%CI = 1.27 - 2.76; p = 0.0015). Six other SNPs showed a trend toward association with hypospadias. The *DGKK* nucleotide variants demonstrated moderate linkage disequilibrium (LD). D’ and r2 values, calculated from the genotype data of the control samples, ranged from 0.607 to 1.000 and 0.131 to 0.984, respectively (Figure 1 and Table 4).

Subsequently, we tested the common *DGKK* haplotypes for their association with the risk of hypospadias. The global p-values for the two two-markers haplotypes (rs11091748\_rs17328236, rs1934179\_rs4554617), the one three-markers haplotype (rs1934183\_rs1934179\_rs4554617), and the one four-markers haplotype (rs12171755\_rs1934183\_rs1934179\_rs4554617) were statistically significant even after permutation correction. Detailed analysis of those haplotypes is presented in Table 5. All tested SNPs were involved in haplotype combinations associated with hypospadias. However, the haplotype combination (rs1934179\_rs4554617) with the best global p-value (pcorr = 0.007) does not include the two SNPs (rs11091748 and rs12171755) highly linked with hypospadias in the single markers analysis (Table 2).

Discussion

Identifying the major genetic alternations leading to hypospadias will have an impact on genetic counselling and will lead to a greater understanding of the male urinary tract development. Our study builds on previous publications which have reported that the genetic susceptibility of hypospadias may be associated with common variants of the *DGKK* gene [5-8]. In our mono-ethnic sample, the *DGKK* haplotypes were found to be strongly associated with hypospadias and provided further evidence that *DGKK* may be an important disease-promoting gene [10,11,14,15]. The high odds ratios and level of significance provide compelling support for the observed haplotypes associations, despite the small numbers of participants. For the two investigated SNPs, in the presented Polish sample of patients, evidence of association with hypospadias was found only using haplotypes testing. The lack of association in the single marker analysis may be attributed to a lack of power, secondary to small sample size. An alternative explanation might be that the analyzed variants do not target the causal variant in the Polish population adequately, due to the presence of differing haplotypic structures in specific mono-ethnic populations [4,10,14]. In accordance with our study, Carmichael et al. [7] have previously found evidence of association between two blocks of *DGKK* haplotypes and the hypospadias risk in Californian population. In their study, an 8-SNPs block contained rs12171755, rs19341179 and rs19341179, which were also associated with increased risk of being born with hypospadias in the Polish population. In contrast to our results, Ma et al. [8] did not observe the association between *DGKK* haplotypes and hypospadias susceptibility in the Han Chinese population. These findings support the assumption that the functional variants associated with these risky SNPs of *DGKK* are likely to be regulatory in nature. More in-depth investigations are necessary to explore the functional and mechanistic role of *DGKK* in the male urinary system. Rigorously establishing the genetic risk for any multifactorial disorder is important but inherently difficult [4,10].

Our study represents a step forward in understanding the genetic basis of isolated hypospadias. The study provides strong evidence of an association of *DGKK* haplotypes with the susceptibility to hypospadias. Further testing in independent populations and meta-analyses are needed to clarify the role of nominally significant polymorphic variants of the *DGKK* gene association with hypospadias.

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**Author contributions**

This work was conducted as a collaboration between all the authors (KKH: literature search, design of the study, patient selection, data interpretation, writing the manuscript; AM: design of the study, genotyping, data interpretation; AK and DM: patient selection, data interpretation; AT: genotyping, data interpretation; PPJ: data interpretation). All authors read and approved the final manuscript.

**Conflict of interest statement**

The authors state that there are no conflicts of interest regarding the publication of this article.

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