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EVC gene polymorphisms and risks of isolated hypospadias – a preliminary study

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Introduction Hypospadias has a complex etiology with both genetic and environmental factors contributing to the condition. Urogenital abnormalities including hypospadias, are found in 22% of cases with Ellis van Creveld syndrome (EvC). Mutations in the *EVC* gene can cause major and minor anomalies, which form phenotypes that partially overlap with those present in EvC.
The aim of this study was to evaluate the association between nucleotide variants of the *EVC* gene and the risk of hypospadias.
Material and methods Four single nucleotide polymorphisms (SNPs) of the *EVC* gene (rs3774856, rs2302075, rs1383180, rs7680768) were taken under investigation in 96 patients with isolated hypospadias and 284 matched controls. Genotyping of all polymorphisms was carried out by PCR and followed by appropriate restriction enzyme digestion (PCR-RFLP).
Results Individuals homozygous for the SNP rs2302075 (p.Thr449Lys) showed an elevated risk for hypospadias. Haplotypes containing the rs2302075 variant also revealed modest associations with hypospadias, which did not survive multiple testing corrections. None of the other tested *EVC* polymorphisms displayed significant association with the risk of hypospadias, either in dominant or recessive inheritance models.
Conclusions The results of this study suggest that polymorphic variants of the *EVC* gene do not substantially contribute to the risk of hypospadias based on our study population. However, further studies should help to clarify the relationship between polymorphisms of *EVC* and hypospadias.

Key Words: hypospadias ‹› Ellis-van Creveld syndrome ‹› gene polymorphisms

INTRODUCTION

Hypospadias is one of the most common congenital disorders in males [1]. Anterior (glandular) and middle (penile) form of hypospadias are presented in 70-80% and 15-20% of cases, respectively. Posterior (penoscrotal, scrotal or perineal) openings are very rare [1, 2]. The more severe degrees are more likely to be associated with chordee or cryptorchidism. Hypospadias has an effect on 1:200-1:300 male births [1]. Recently, the prevalence in Poland was estimated to be 17.9 per 10 000 births, which was similar to the European statistic of 18.1 per 10000 births (EUROCAT). Hypospadias has a complex etiology with both genetic and environmental factors contributing to this condition [3]. Well known candidate genes, which are correlated with the risk of hypospadias in various populations, include *DGKK, FGF10, BMP7, SHH, CYP3A4* and *MAMLD1* [3, 4].
Ellis van Creveld syndrome (EvC; OMIM 225500) is an autosomal recessive chondro-epidermal dysplasia, which was first described in 1940 by Richard Ellis and Simon van Creveld [5]. The incidence of EvC in the general population is estimated to be

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at 0.7 per 100 000 live births, but it is more prevalent in the United Arab Emirates (5.2 per 100 000 live births) and in the Amish population of Lancaster County, Pennsylvania, USA (5 per 1 000 live births) [6]. It is characterized by disproportionate dwarfism, with short ribs, limbs, post-axial polydactyly, and dysplastic nails, teeth, as well as, heart defects [6]. Urogenital abnormalities, like renal agenesis or dysplasia, megaureter, nephrocalcinosis, cryptorchidism, and hypospadias were found in 22% of cases [7, 8]. Mutations in *EVC* and *EVC2* genes, located in the head-to-head configuration on chromosome 4p16, have been identified as causative. Alterations in those genes also cause other major and minor anomalies. These alterations form phenotypes that partially overlap with EvC and an autosomal dominant disorder called Weyers acrofacial dysostosis [9, 10]. The clinical presentation of patients with mutations in *EVC versus EVC2* genes is indistinguishable. The *EVC* gene encodes a 992-amino acid protein that contains a transmembrane domain, 3 nuclear localization signals and a leucine zipper motif. The EVC protein is localized at the chondrocyte cilia base and is an intracellular component of the Hedgehog (Hh) signaling pathway, which is required for transcriptional activation of the Indian Hh Pathway [9]. Hh proteins are major developing regulators during embryogenesis and they interact with various signaling molecules to promote cell proliferation, survival and differentiation [11]. Recent studies have revealed the possible involvement of one of the Hh proteins, Sonic Hedgehog, in early genital tubercle outgrowth and patterning [9, 11]. Sonic Hedgehog staining was the greatest in the urethral epithelium at 14 weeks gestation, correlating with the time of urethral tubularization [12].

Such findings encourage us to search for a correlation between single nucleotide polymorphisms (SNPs) in the *EVC* gene and non-syndromic hypospadias.

MATERIAL AND METHODS

Material

The patients included 96 boys with isolated glandular and middle forms of hypospadias not involving cryptorchidism. They were recruited into the study from the Department of Pediatric Surgery at the Institute of Mother and Child in Warsaw. Case eligibility was determined using detailed medical records. The isolated designation was based on the diagnosis of hypospadias with no other apparent structural anomalies. The control group was comprised of 284 healthy, non-related boys with no family history of hypospadias or other congenital structural anomalies, whom were mostly patients attending local primary care pediatricians and general practitioners. All unrelated participants were Caucasians of Polish origin, born in Poland. Written and oral consent was obtained from the legal guardians of all the participants. The procedures of the study were approved by the Local Ethical Committee of the Institute of Mother and Child.

SNP selection and genotyping

Genomic DNA was isolated from peripheral blood lymphocytes by a salt-out extraction procedure. SNPs in the *EVC* gene were identified from the HapMap Genome Browser (http://hapmap. ncbi.nlm.nih.gov/), the NCBI dbSNP database (http://www.ncbi.nlm.nih.gov/projects/SNP/) and related literature. A final set of 4 SNPs was selected based on a minor allele frequency (MAF) over 15% in the Caucasian population and the *EVC* gene-linkage disequilibrium (LD) pattern. Characteristics of SNPs that were finally selected are presented in Table 1. The LD pattern and the structure of the haplotype blocks across the *EVC* gene were determined using genotype data from the HapMap database and Haploview 4.0 software (http://www.broad.mit.edu/mpg/haploview/). The plot of the pairwise LD between SNPs in the *EVC* gene is presented in Figure 1.

Genotyping of all polymorphisms was carried out by PCR and followed by appropriate restriction enzyme digestion (PCR-RFLP) according to the manufacturer's instructions (New England Biolabs, Ipswich, England). DNA fragments were separated using electrophoresis on 2% agarose gel and visualized using ethidium bromide staining. Primer sequences and conditions for PCR-RFLP analyses are presented in Table 2. For quality control, approximately 10% of the randomly chosen samples were re-genotyped. Samples that failed the genotyping were excluded from statistical analyses. Laboratory technicians were blinded to the case/control status of all samples and to the inclusion of duplicate samples for quality control.

Statistical analysis

Deviation from the Hardy-Weinberg equilibrium (HWE) for all SNPs was tested in both patients and controls using the chi-square (χ^2) test. Statistically significant deviations from the HWE expectations was interpreted as a p-value <0.05. The differences in allele and genotype frequencies between cases and controls were determined using the standard χ^2 and Fisher exact tests. SNPs were tested for association with hypospadias using the Cochran-Armitage trend test. The odds ratio (OR) and associated 95% confidence intervals (95% CI) for patients versus

controls were also calculated. The dominant and recessive models were analysed. The Bonferroni correction for multiple comparisons was applied to the p-values (alpha level p = 0.0125). Haplotype based association analysis using a sliding window approach was performed using the Haploview 4.2 software. Statistical significance was assessed using the 1,000-fold permutation test.

RESULTS

The sample success rate was on average 99.2% for the genotyped SNPs and the concordance rate was 100% according to the duplicate analysis. None of the tested polymorphisms in the cases or controls showed evidence of deviation from the HW equilibrium The MAF for all SNPs was at least 23%. The genotyping results, OR, and 95% CI calculations, for the 4 SNPs of the *EVC* are reported in Table 3. Using the recessive genetic model, there was a borderline association between rs2302075 variant and the risk for hypospadias. Compared to individuals with the GT or TT genotype, the GG homozygotes had an OR of 2.011 (95% CI: 0.879 – 4.601; p = 0.093). There was no significant association of rs2302075 with hypospadias under the dominant genetic model (Table 3). None of the other three *EVC* polymorphisms displayed a significant association

Table 1. *Characteristics of polymorphisms genotyped in the EVC gene*

rs no.	Location	Alleles	SNP function[a]	Protein effect	MAF[b]
rs3774856	chr4:5713910	A/G (FWD)	intron		0.23
rs2302075	chr4:5755542	G/T (REV)	missense	p.Thr449Lys	0.23
rs1383180	chr4:5785442	C/T (REV)	missense	p.Arg576Gln	0.42
rs7680768	chr4:5809187	A/G (FWD)	intron		0.46

[a]According to the Single Nucleotide Polymorphism database (dbSNP); [b]MAF, minor allele frequency calculated from the control samples; FWD, forward; REV, reverse strand

Table 2. *RFLP conditions for the identification of polymorphisms genotyped in the EVC gene*

rs no.	Alleles[a]	Primers for PCR amplification (5′ – 3′)	Annealing temp. (°C)	PCR product length (bp)	Restriction enzyme	Restriction fragment length (bp)
rs3774856	A/g	F: CAAGGAGAAGGACGAATTGC R: GCCACTTGCATAGGAAGCAT	62.6	324	BsrI	A = 215 + 81 + 28 G = 296 + 28
rs2302075	g/T	F: CTCAAGACGTGGAGGCATCT R: TGTAGGGGCTAAGGGACTGA	66.3	519	EcoNI	G = 317 + 202 T = 236 + 202 + 81
rs1383180	C/t	F: GTGTCTTGTGGGAGGCTTGT R: CGACTTCCTGTTGAGGGAGA	67.0	514	MspI	C = 251 + 190 + 73 T = 324 + 190
rs7680768	A/g	F: TGTGGGTCTCTGTTCACACC R: CCTCCGTTCCTAAGCAGTCA	67.0	416	XbaI	A = 297 + 120 G = 416

RFLP – Restriction Fragment Length Polymorphism analysis; [a]Uppercase denotes the more frequent allele in the control samples

Table 3. *Association of EVC gene SNPs with the risk of hypospadias*

rs no.	Alleles[a]	MAF[b]	Genotypes cases[c]	Genotypes controls[c]	p_{trend} value	$p_{genotypic}$ value	$p_{allelic}$ value	$OR_{dominant}$ (95% CI)[d]; p value	$OR_{recessive}$ (95% CI)[e]; p value
rs3774856	A/g	0,23	4/30/61	13/103/167	0.417	0.667	0.422	0.802 (0.496 – 1.299); 0.370	0.913 (0.290 – 2.871); 1.000[f]
rs2302075	g/T	0,23	10/29/54	16/99/168	0.390	0.229	0.375	1.055 (0.656 – 1.697); 0.825	2.011 (0.879 – 4.601); 0.093
rs1383180	C/t	0,42	22/47/27	56/127/101	0.214	0.403	0.199	1.410 (0.850 – 2.342); 0.183	1.210 (0.692 – 2.116); 0.502
rs7680768	A/g	0,46	23/47/25	61/138/83	0.499	0.796	0.496	1.168 (0.692 – 1.972); 0.561	1.157 (0.669 – 2.003); 0.601

[a]Uppercase denotes the more frequent allele in the control samples; [b]MAF – minor allele frequency calculated from the control samples; [c]The order of genotypes: dd / Dd / DD (d is the minor allele); [d]Dominant model: dd + Dd *vs.* DD (d is the minor allele); [e]Recessive model: dd *vs.* Dd + DD (d is the minor allele); [f]Fisher exact test

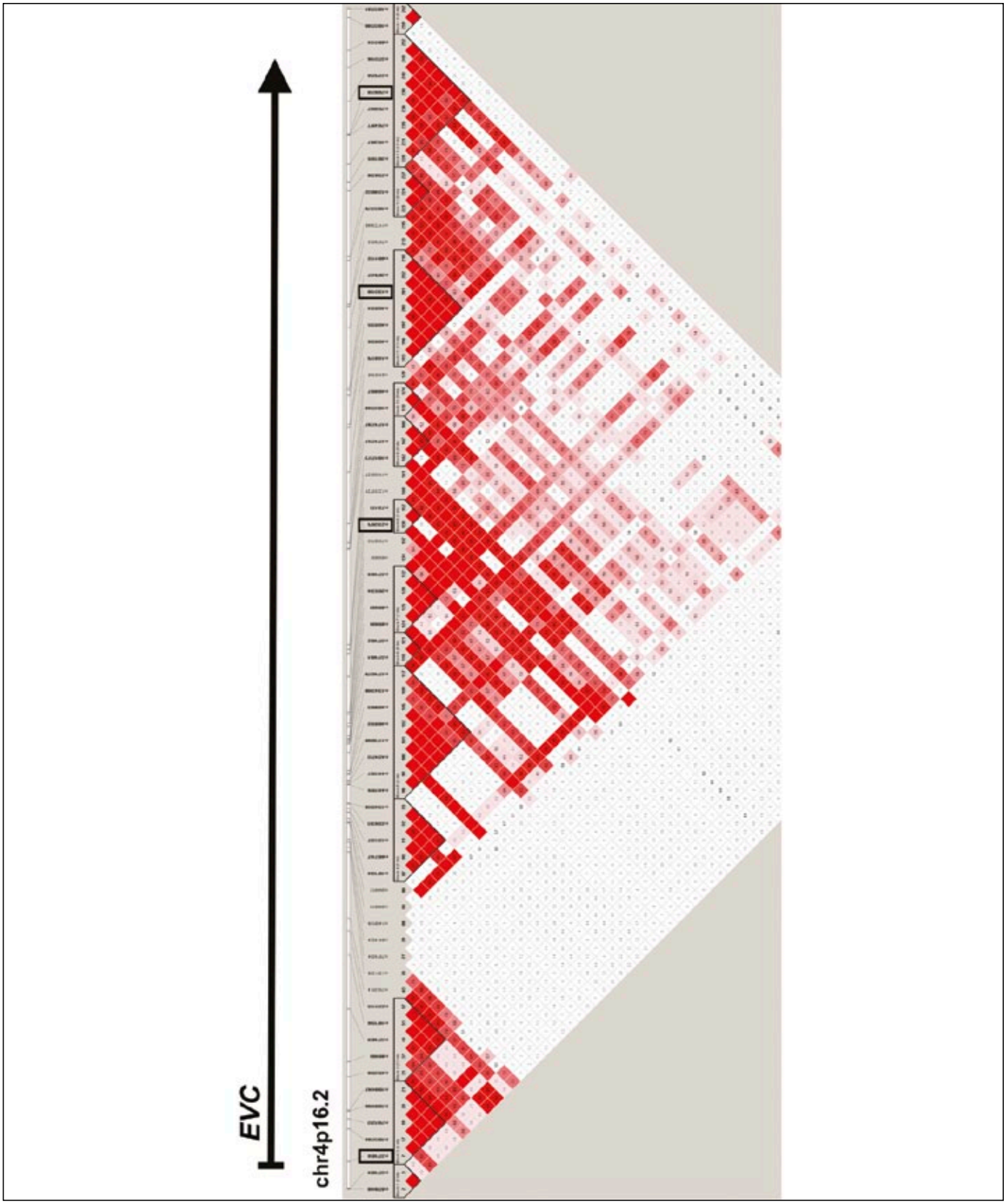

Figure 1. *The Linkage Disequilibrium (LD) plot of HapMap SNPs within the EVC region. The plot was generated using the genotype data from HapMap CEU samples and the Haploview 4.0 software (Broad Institute, Cambridge, MA). The names of the tested SNPs are enclosed in boxes. The numbers in the squares indicate percentage of LD between a given pair of SNPs (D' values).*

with the risk of hypospadias in either the dominant or recessive inheritance models (Table 3).
The study of haplotype effects has identified the borderline association between the T-C (rs2302075_rs1383180), A-T-C (rs3774856_rs2302075_rs1383180) haplotypes and hypospadias (Table 4). There was no evidence of statistically significant differences in the distribution of haplotypes not comprising of the rs2302075 variant between the cases and controls.

DISCUSSION

Urogenital health has been subject to increasing interest and concern during recent years. Although hypospadias is a very common anomaly which can cause life-long problems with emotional development and social integration, its etiology still remains unclear [1, 13]. So far, the majority of studies were concentrated on genes involved in the genital tubercle formation, androgen dependent sexual differentiation and transcription factors [3, 4].To the best of our knowledge, this study is the first to examine haplotypes and polymorphisms in the *EVC* gene with respect to hypospadias. The SNP rs2302075 tended to associate with the risk of hypospadias in our study population. An association of this SNP is plausible, given that the study of haplotype effects suggested its involvement. The present data suggests that the other examined SNPs had no influence on the hypospadias risk. Nonetheless, it will be interesting to see the results of other studies

Table 4. *Haplotype analysis of SNPs genotyped in the EVC gene*

Polymorphisms	Haplotypes	Frequency	Case, Control Ratios	χ^2	p value	p_{corr} value
rs3774856_rs2302075	AT	0.589	0.581, 0.592	0.072	0.789	0.993
	AG	0.190	0.219, 0.180	1.393	0.238	0.539
	GT	0.172	0.156, 0.177	0.413	0.521	0.884
	GG	0.049	0.044, 0.051	0.169	0.681	0.971
rs2302075_rs1383180	TT	0.410	0.444, 0.398	1.247	0.264	0.609
	TC	0.350	0.291, 0.371	4.026	0.045	0.118
	GC	0.215	0.235, 0.208	0.625	0.429	0.821
	GT	0.025	0.030, 0.023	0.286	0.593	0.937
rs1383180_rs7680768	CA	0.309	0.280, 0.319	0.991	0.320	0.660
	CG	0.257	0.246, 0.261	0.164	0.685	0.962
	TA	0.223	0.231, 0.220	0.088	0.767	0.985
	TG	0.211	0.243, 0.200	1.584	0.208	0.477
rs3774856_rs2302075_rs1383180	ATT	0.311	0.361, 0.295	2.980	0.084	0.280
	ATC	0.278	0.219, 0.298	4.450	0.035	0.104
	AGC	0.174	0.198, 0.165	1.106	0.293	0.799
	GTT	0.099	0.083, 0.104	0.683	0.408	0.922
	GTC	0.072	0.071, 0.072	0.004	0.952	1.000
	GGC	0.042	0.037, 0.043	0.120	0.729	0.999
	AGT	0.016	0.021, 0.014	0.382	0.537	0.983
rs2302075_rs1383180_rs7680768	TTA	0.209	0.210, 0.209	0.003	0.960	1.000
	TTG	0.201	0.234, 0.190	1.740	0.187	0.620
	TCA	0.180	0.148, 0.190	1.737	0.188	0.621
	TCG	0.171	0.143, 0.180	1.409	0.235	0.710
	GCA	0.128	0.132, 0.127	0.028	0.868	1.000
	GCG	0.086	0.103, 0.081	0.889	0.346	0.890
	GTA	0.015	0.021, 0.013	0.627	0.428	0.949
	GTG	0.010	0.009, 0.010	0.015	0.904	1.000
rs3774856_rs2302075_rs1383180_rs7680768	ATTG	0.164	0.197, 0.153	1.963	0.161	0.562
	ATCA	0.156	0.129, 0.165	1.460	0.227	0.761
	ATTA	0.151	0.168, 0.145	0.585	0.444	0.989
	ATCG	0.119	0.087, 0.129	2.505	0.114	0.397
	AGCA	0.100	0.113, 0.095	0.488	0.485	0.997
	AGCG	0.074	0.087, 0.070	0.644	0.422	0.982
	GTTA	0.057	0.038, 0.064	1.774	0.183	0.634
	GTCG	0.052	0.053, 0.052	0.004	0.950	1.000
	GTTG	0.038	0.042, 0.037	0.116	0.733	1.000
	GGCA	0.029	0.020, 0.032	0.688	0.407	0.977
	GTCA	0.023	0.022, 0.024	0.018	0.895	1.000
	GGCG	0.012	0.015, 0.011	0.162	0.688	0.999

[a]p value calculated using permutation test and a total of 1,000 permutations.

analyzing associations between *EVC* and hypospadias. The Estonian study of Must et al. [14], revealed a contribution of *EVC* rs1383180, but not rs2302075, to a susceptibility to suicide only in males, which could suggest an interplay between *EVC* and sex hormones. Recently, strong association between *EVC* rs1383180 and smoking-related pancreatic cancer was reported [15]. Results from some studies indexed in PubMed showed positive correlation between maternal smoking and hypospadias in offspring, although the majority of papers pointed towards no association [16]. Interestingly, the only two haplotypes, which tended to be associated with hypospadias in our study population, was comprised of the minor alleles rs2302075 and rs1383180. The impact of the rs2302075 polymorphism, leading to amino acid Thr449Lys substitution on EVC activity in tissues, remains unclear. It is noteworthy, that both rs1383180 and rs2302075 polymorphic variants had been found to be informative in a large family manifesting atypical EvC, in which an etiological *EVC* mutation was found [10].

The major limitation of this preliminary study was the sample size, which did not allow us to detect disease predisposing variants with small or modest effect. We must also note that the number of selected polymorphisms does not cover the *EVC* gene fully. Lastly, given the number of comparisons that we performed, we cannot exclude the possibility that all the observed borderline associations occurred by chance. This study also had a number of strengths. Our case and control populations were ethnically homogenous. In addition, we were able to establish an isolated hypospadias phenotype in all cases.

CONCLUSIONS

In conclusion, these results provide very limited evidence that the variation in the *EVC* gene is associated with a risk of clinically mild forms of hypospadias. The borderline significance of all reported associations necessitate caution in their interpretation. Identification of genetic factors underlying the etiology of hypospadias is crucial for improving prevention strategies and genetic risk counselling. Further studies should help to clarify the relationship between polymorphic variants of *EVC* and the risks of this common anomaly.

CONFLICTS OF INTEREST

The authors declare no conflicts of interest.

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