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

Andrzej Kowal¹, Adrianna Mostowska², Dariusz Mydlak¹, Bożena Eberdt-Goląbek³, Matthew Misztal², Paweł P. Jagodziński², Kamil K. Hozyasz³

¹Department of Pediatric Surgery, Institute of Mother and Child, Warsaw, Poland
²Department of Biochemistry and Molecular Biology, Poznań University of Medical Sciences, Poznań, Poland
³Department of Pediatrics, Institute of Mother and Child, Warsaw, Poland

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
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 Haplovievew 4.0 software (http://www.broad.mit.edu/mpg/haplovieview/). 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 ($\chi^2$) 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 $\chi^2$ 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 | Protein effect | MAF* |
|---------|----------|---------|--------------|----------------|------|
| rs3774856 | chr4:5713910 | A/G (FWD) | intron | p.Thr449Lys | 0.23 |
| rs2302075 | chr4:5755542 | G/T (REV) | missense |  | 0.23 |
| rs1383180 | chr4:5785442 | C/T (REV) | missense | p.Arg766Gln | 0.42 |
| rs7680768 | chr4:5809187 | A/G (FWD) | intron |  | 0.46 |

*According to the Single Nucleotide Polymorphism database (dbSNP); **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* | Primers for PCR amplification (5’ – 3’) | Annealing temp. (°C) | PCR product length (bp) | Restriction enzyme | Restriction fragment length (bp) |
|---------|---------|------------------------------------------|----------------------|------------------------|-------------------|----------------------------------|
| rs3774856 | A/g | F: CAAGGAGAAGGAGCAATTGC R: GCCACTTGCTAGAGGACAT | 62.6 | 324 | BsrI | A = 215 + 81 + 28 G = 296 + 28 |
| rs2302075 | g/T | F: CTCAAGCGTGAGGCACTCT R: TGAGGGGCTAAGGGACTGA | 66.3 | 519 | EcoNI | G = 317 + 202 T = 236 + 202 + 81 |
| rs1383180 | C/t | F: GTGTCTTGGGAGGCTTTG R: CGACTTCTGGTTAGGGAGGA | 67.0 | 514 | MspI | C = 251 + 190 + 73 T = 324 + 190 |
| rs7680768 | A/g | F: TGTTGCTGTCGGGCTCCCA R: CTCGGTTCCTAAGCAGTCA | 67.0 | 416 | XbaI | A = 297 + 120 G = 416 |

RFLP – Restriction Fragment Length Polymorphism analysis; *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* | MAF* | Genotypes cases | Genotypes controls | p_{trend} | p_{genotypic} | p_{allelic} | OR_{dominant} (95% CI)*; p value | OR_{recessive} (95% CI)*; 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 |
| 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 |

*Uppercase denotes the more frequent allele in the control samples; **MAF – minor allele frequency calculated from the control samples; *The order of genotypes: dd / Dd / DD (d is the minor allele); **Dominant model: dd / Dd vs. DD (d is the minor allele); *Recessive model: dd vs. Dd + DD (d is the minor allele); *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 | $\chi^2$ | 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 |
| | GTC | 0.099 | 0.083, 0.104 | 0.683 | 0.408 | 0.922 |
| | GTG | 0.072 | 0.071, 0.072 | 0.004 | 0.952 | 1.000 |
| | GCC | 0.042 | 0.037, 0.043 | 0.120 | 0.729 | 0.999 |
| | GTC | 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 |
| | GCC | 0.086 | 0.103, 0.081 | 0.889 | 0.346 | 0.890 |
| | GTC | 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 |
| | GGGG | 0.012 | 0.015, 0.011 | 0.162 | 0.688 | 0.999 |

*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 homogeneous. 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.

ACKNOWLEDGMENTS

The technical assistance of MSc Sylwia Matuszewska, Mrs Katarzyna Szwedkowicz and Mrs Ewa Pietrzak. This work was supported by grant No 510-05-52 from Institute of Mother and Child.

References

1. Stein R. Hypospadias. Eur Urol Suppl. 2012; 11: 33–45.
2. Genc A, Taneli C, Oksel F, Balkan C, Bilgi Y. Analysis of meatal location in 300 boys. Int Urol Nephrol. 2001; 33: 663-664.
3. van der Zanden LF, van Rooij IA, Feitz WF, Franke B, Koers NV, Roeleveld N. Aetiology of hypospadias: a systematic review of genes and environment. Hum Reprod Update. 2012; 18: 260-283.
4. Carmichael SL, Witte JS, Ma C, Lammer EJ, Shaw GM. Hypospadias and variants in genes related to sex hormone biosynthesis and metabolism. Andrology. 2014; 2: 130-137.
5. Ellis RW, van Crevel S. A syndrome characterized by ectodermal dysplasia, polydactyly, chondro-dysplasia and congenital morbus cordis: report of three cases. Arch Dis Child. 1940; 15: 65-84.
6. Baujat G, Le Merrer M. Ellis-van Crevel syndome. Orphanet J Rare Dis. 2007; 2: 27.
7. Khan I, Ahmed SA, Mohsin K. Ellis-van Creveld syndrome. A case report. J Pak Assoc Dermatol. 2006; 16: 239-242.
8. Babu TA, Biswal N. Are Ellis-van Creveld syndrome cases predisposed to recurrent sepsis? Indian J Med Specialties. 2012; 3: 71-74.
9. D’Asdia MC, Torrente I, Consoli F, et al. Novel and recurrent EVC and EVC2 mutations in Ellis-van Creveld syndrome and Weyers acrofacial dysostosis. Eur J Med Genet. 2013; 56: 80-87.
10. Ulucan H, Gül D, Sapp JC, Cockheram J, Johnston JJ, Biesecker LG. Extending the spectrum of Ellis van Creveld syndrome: a large family with a mild mutation in the EVC gene. BMC Med Genet. 2008; 9: 92.
11. Pusapati GV, Hughes CE, Dorn KV, VB, et al. EF.CAB7 and IQCE regulate Hedgehog signaling by tethering the EVC-EVC2 complex to the base of primary cilia. Dev Cell. 2014; 28: 483-496.
12. Shehata BM, Elmore JM, Bootwala Y, et al. Immunohistochemical characterization of sonic hedgehog and its downstream signaling molecules during human penile development. Fetal Pediatr Pathol. 2011; 30: 244-251.
13. Jiao C, Wu R, Xu X, Yu Q. Long-term outcome of penile appearance and sexual function after hypospadias repairs: situatation and relation. Int Urol Nephrol. 2011; 43: 47-54.
14. Must A, Kõks S, Vasar E, et al. Common variations in 4p locus are related to male completed suicide. Neuromolecular Med. 2009; 11: 13-19.
15. Tang H, Wei P, Duell EJC, et al. Axonal guidance signalling pathway interacting with smoking in modifying the risk of pancreatic cancer: a gene- and pathway-based interaction analysis of GWAS data. Carcinogenesis. 2014; 35: 1039-1045.
16. Håkonsen LB, Ernst A, Ramlau-Hansen CH. Maternal cigarette smoking during pregnancy and reproductive health in children: a review of epidemiological studies. Asian J Androl. 2014; 16: 39-49.