Genomic Variants of *ATF3* in Patients With Hypospadias

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Purpose: *ATF3*, an estrogen responsive gene expressed during genital development, could be implicated in the etiology of hypospadias. *ATF3* is up-regulated in the foreskin of patients with hypospadias and is implicated in suppression of the cell cycle, which may interfere with urethral cell growth. We sought to investigate the sequence of *ATF3* in patients with hypospadias.

Material and Methods: Direct sequencing of coding exons and splice sites of *ATF3* was performed in 41 boys with hypospadias and 30 controls. In addition, ATF3 expression in 1 human fetal penis with and 1 without hypospadias was studied by immunohistochemical analysis.

Results: A missense variant (L23M) was identified in a boy with anterior hypospadias. This amino acid is highly conserved. Three genomic variants (C53070T, C53632A, Ins53943A) were found in or close to exon 6 in patients with perineal, penoscrotal and anterior hypospadias. This important exon includes splice sites for an alternative transcript (*ATF3*ΔZip) that have been implicated in regulation of the function of *ATF3*. None of these genomic variants was present in controls. Immunochemical analysis revealed that in normal fetuses ATF3 is not expressed in and around the urethra, while in patients with hypospadias ATF3 is over expressed in the urethral plate and subcutaneous tissue, especially around the ectopic orifice of the urethra.

Conclusions: Genomic variants of *ATF3* are present in 10% of our patients with hypospadias. We also report an abnormal expression pattern of ATF3 in a hypospadiac fetus. The direct implication of ATF3 in the occurrence of hypospadias remains to be confirmed by functional studies of the genomic variants we describe.

Key Words: activating transcription factor 3, genetics, hypospadias, sex differentiation, urogenital abnormalities

Hypospadias is one of the most common congenital defects in humans. European studies have documented an unexplained increase in the occurrence of hypospadias from the 1970s to the 1990s.[1] In addition, 2 birth defect data surveillance systems in the United States have identified an unexplained doubling in the number of cases of this abnormality in the last 30 years.[2,3] The molecular events required in the genitourinary tract for normal development of the external genitalia are just beginning to be elucidated.[4,5] Research findings suggest that male urethral development involves fusion of the urethral folds followed by removal of the epithelial seam.[6] This process is complex and requires genetic programming, hormone signaling and tissue remodeling in a sequential fashion. A disturbance in one of these processes may lead to hypospadias.

Endocrine disruptors have been proposed as a possible explanation for the increasing incidence of hypospadias in industrialized countries.[4] Microarray work and more targeted expression studies have indicated that *ATF3* (Gene ID 467 at http://www.ncbi.nlm.nih.gov/sites/entrez) is an excellent candidate gene for hypospadias, since it is up-regulated in human hypospadiac tissue compared to normal circumcision controls, and it is estrogen responsive in vitro and in vivo.[7–9]

Several hypotheses could be proposed regarding these findings. First, our repeated confirmation of up-regulated expression of *ATF3* and the role of estrogen in the development of hypospadias led directly to an interesting molecular intersection where ATF3 expression is regulated by estrogens.[10,11] Indeed, ATF3 might lie at the center of the interactions between the TGF-β signaling pathway (involved in mouse genital tubercle development) and steroid hormone receptors.[12,13] In epithelial cells *ATF3* responds to a given signal via the TGF-β pathway, in which TGF-β facilitates epithelial and mesenchymal differentiation by suppressing expression of the inhibitor of DNA binding 1 (*ID1*) gene.[12] Furthermore, an increase in TGF-β results in an increase in estrogen receptor transcripts.[14] Increased numbers of estrogen receptors might allow increased estrogen signaling in the presence of exogenous estrogens, and application of exogenous estrogens results in up-regulation of ATF3, which potentially leads to hypospadias.

In addition, variations of *ATF3* expression could implicate genetic variants of the gene affecting the regulation of its activity and the isoform ATF3ΔZip.[15] Lastly, this variation of expression of *ATF3* could be not only the consequence of exposure to xenoestrogens, but also the evidence of a

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particular susceptibility to this exposure. Indeed, susceptibility to environmental factors might depend not only on the endocrine disruptor itself, but also on individual sensitivity, which is modulated by genetic background, including polymorphisms.

To our knowledge no previous study has sought genetic variants in the *ATF3* gene in patients with hypospadias of varying severity. Because of the relationships between *ATF3* expression in human foreskin and hypospadias, we screened for mutations of this gene in patients with this malformation and characterized the expression profile of *ATF3* at the malformation site, the urethral plate of normal and hypospadiac human fetuses.

MATERIALS AND METHODS

Patients and Controls

A total of 71 individuals 7 months to 6 years old were included in this study. Of the patients 41 presented with isolated hypospadias. Clinical severity ranged from glanular to perineal hypospadias. The urethral meatus was glanular in 3 patients, subcoronal in 3, distal in 5, mid shaft in 4, proximal in 8, penoscrotal in 14 and perineal in 3. Patients with abnormal plasma testosterone level were excluded from this study. Controls consisted of 30 normal individuals who underwent circumcision for phimosis resistant to local corticoid treatment or due to elective parental preference. Written consent was obtained for all patients.

DNA Extraction

Excess skin at the time of hypospadias surgery and/or circumcision was frozen in liquid nitrogen. DNA was extracted from this tissue using DNAzol®. The manufacturer protocol for DNA isolation was followed, with minor modifications. Briefly, 0.5 ml DNAzol was added to the sample and gently ground to ensure proper homogenization.

Tubes were centrifuged for 10 minutes at 10,500 g to pellet cell debris. Supernatant fluids were transferred to new 1.7 ml tubes followed by the addition of 0.5 ml 100% ethanol. Tubes were inverted several times and then allowed to stand for 15 to 30 minutes at 4C to precipitate the DNA. Precipitated DNA was pelleted by centrifuging for 2 minutes at 5,000 g and supernatant fluids were removed using a vacuum suction device. The DNA was washed by adding 0.8 ml 95% ethanol to the tubes, inverting the tubes to resuspend the pellets and then centrifuging the tubes for 2 minutes at 5,000 g to re-pellet the DNA. Supernatant fluids were removed by suction and the wash step was repeated. The final DNA pellets were dissolved in 40 μl 8 mM NaOH buffer.

Mutational Analysis

Direct sequencing of the coding exons of *ATF3* and their flanking splice sites was performed. Primers are summarized in the Appendix. The cycling protocol was identical for all exons and consisted of an initial denaturation step at 93 C (2 minutes), followed by 39 cycles of denaturation at 93 C (30 seconds), hybridization at 58 C (30 seconds) and extension at 72 C (30 seconds), with a final extension step of 6 minutes at 72 C. To ensure the absence of contamination, DNA from controls and DNA-free samples were run simultaneously.

All polymerase chain reaction products were analyzed in 2% agarose gel. DNA sequencing was performed with the antisense and/or sense primer. The 3730xl DNA Analyzer (Applied Biosystems, Foster City, California) was used. Sequencing reactions were repeated twice with at least 2 different polymerase chain reaction products. The DNA sequences were compared to the sequences of normal controls and to the reference genome from the Ensembl database (www.ensembl.org) and the GenBank® database (ID 467 at http://www.ncbi.nlm.nih.gov).

Homology Studies

Putative homologues of human *ATF3* were detected by the Ensembl database and HomoloGene (www.ncbi.nlm.nih.gov). The alignments were made by Ensembl online alignment and with ClustalW software (www.clustal.org).

Immunochemistry

A normal and a hypospadiac human fetus (21 and 35 weeks of gestation, respectively) were studied. The etiology of the hypospadias in the fetus was unknown. The external genitalia were fixed in formalin, embedded in paraffin wax and serially sectioned at 6 μm from the tip to the base of the penis. Histological specimens were evaluated for expression of ATF3 protein by immunohistochemical staining using the standard protocol accompanying the avidin-biotin-peroxidase complex kit, with overnight primary antibody incubation with rabbit anti-ATF3 at a dilution of 1:400. Negative control sections were incubated without primary antibody and thyroid was used as a positive control. Digital images were acquired with a Leica camera (Leica Camera AG, Solms, Germany) and ACT-1 software (Nikon Inc., Melville, New York).

RESULTS

Mutation Screening

Four genomic variants were identified among patients with hypospadias (9.7%) and none were found in controls. A heterozygous missense mutation in exon 3 (L23M) was identified in a boy with anterior hypospadias (fig. 1, *a*). There was no familial history of genital malformation, and blood from the mother was unavailable. The homology study showed that this amino acid was highly conserved through species (fig. 1, *b*).

Three untranslated genomic variants (C53070T, C53632A, Ins53943A) were also found in patients of various phenotypes with perineal, penoscrotal and anterior hypospadias (fig. 2). These variants occurred in or close to exon 6, near the splice sites for an alternative transcript (*ATF3*ΔZip) implicated in regulation of the function of *ATF3*. There was no familial history of genital malformation.

Previously reported polymorphisms were also identified in patients with hypospadias and controls. The isosense polymorphism Ser68Ser (C49870T) was present in 6 controls (5 heterozygous, 1 homozygous), 4 patients with nonsevere hypospadias (3 heterozygous, 1 homozygous) and 4 patients with severe hypospadias (3 heterozygous, 1 homozygous). The polymorphism T52925C was present in 7 controls (6 heterozygous, 1 homozygous), 6 patients with nonsevere hy-

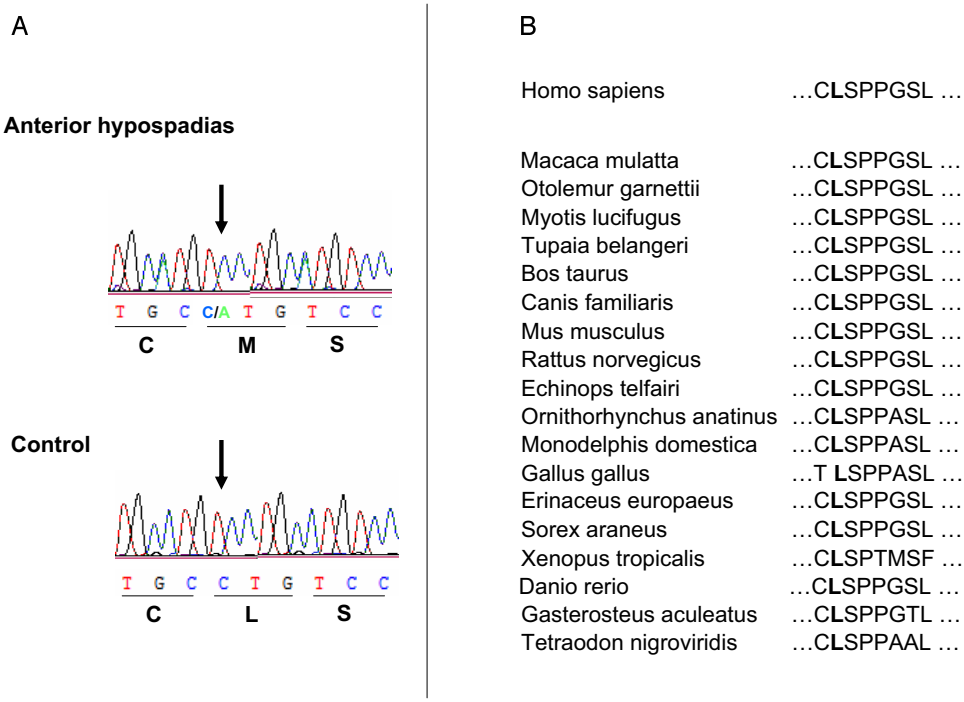

FIG. 1. *a*, chromatogram of L23M heterozygous mutation. Patient presented with isolated hypospadias. *b*, homology study showed this amino acid was highly conserved through species.

pospadias (5 heterozygous, 1 homozygous) and 7 patients with severe hypospadias (6 heterozygous, 1 homozygous). The distribution of these polymorphisms was not significantly different between groups of patients. We did not identify the previously reported polymorphism C38T in our series.

Immunochemistry

Immunochemical analysis of the control fetal specimen demonstrated that ATF3 was not expressed in the proximal and distal urethral plate and subcutaneous tissue of the normal fetus (fig. 3). In contrast, the hypospadiac fetus exhibited a high quantity of ATF3 protein in the urethral plate at the

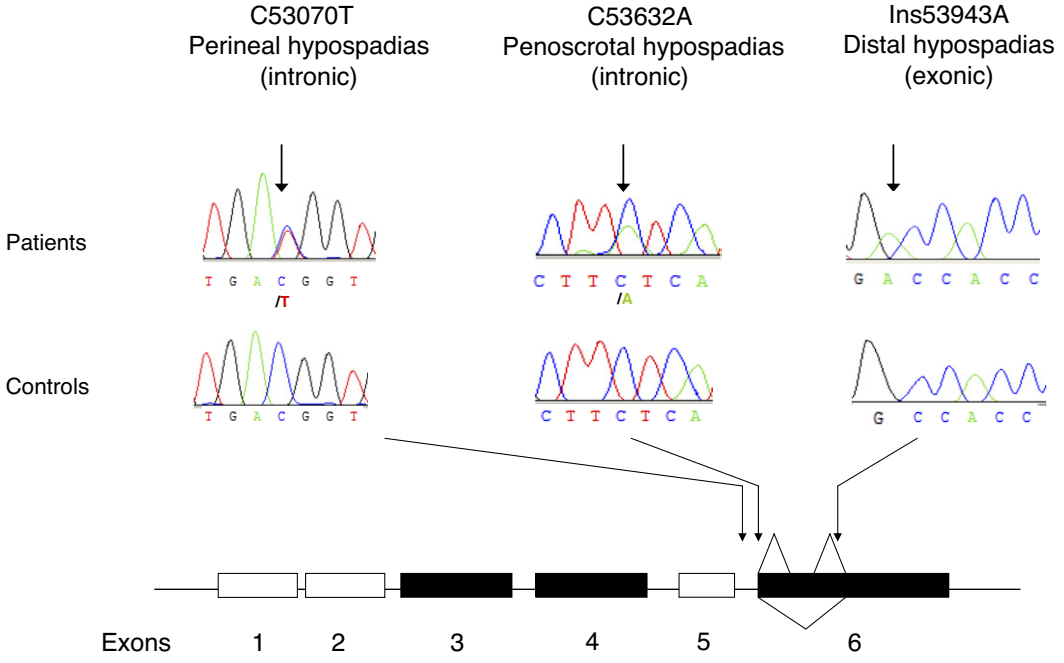

FIG. 2. Chromatograms of patients with hypospadias of varying severity, and controls. These genomic variants were found between or close to splice sites for alternative form of protein ATF3ΔZip, characterized by absence of leucine zipper and implicated in regulation of function of ATF3. White box represents untranslated exon. Black box represents translated exon.

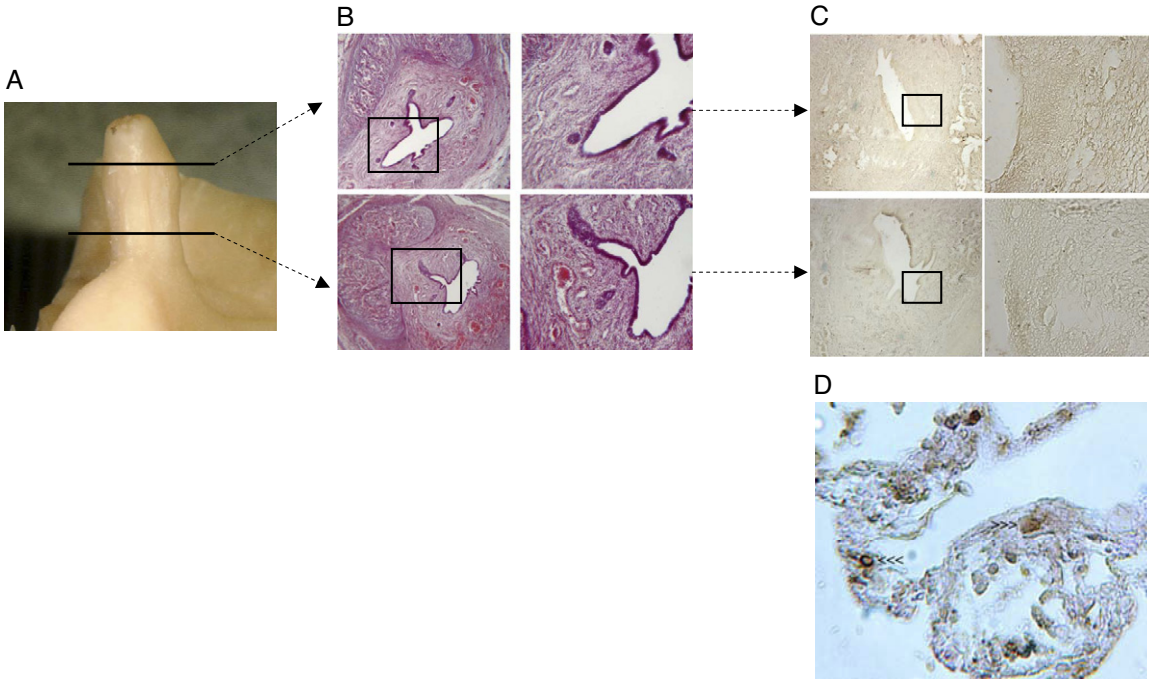

FIG. 3. Immunochemical analysis of ATF3 in normal human genital tubercle (21 weeks of gestation). *a*, macroscopic aspect of specimen. *b*, serial sections of penis stained with hematoxylin and eosin. Left column, reduced from ×100. Right column, reduced from ×200. *c*, immunochemical analysis revealed no significant staining for ATF3, and this result was similar for distal (top photographs) and proximal urethra (bottom photographs). Left column, reduced from ×100. Right column, reduced from ×200. *d*, thyroid was used as positive control. Arrowheads indicate positive nucleus. Reduced from ×400.

level of the ectopic urethral opening (fig. 4). It was noted that this expression was limited to the area of the abnormal urethral opening and was not present in the normal proximal urethra. In respect to cellular location, positive staining was located in the nucleus with congregation to nuclear membrane.

DISCUSSION

Our results reveal that genomic variants of *ATF3* are present in patients with hypospadias of various types. These genomic variants were absent in controls, although the size of the control group was limited in our study. *ATF3* came to our attention as a possible etiology for hypospadias for several reasons.[13] First, microarray analysis of tissues from normal children and patients with hypospadias showed up-regulation of the *ATF3* genes in hypospadias.[7] Also, using a mouse model of steroid hormone dependent genital tubercle development, *Atf3* mRNA levels were increased in all estrogen exposed fetal genital tubercles compared to controls.[9] In addition, immunohistochemical analysis of human foreskin

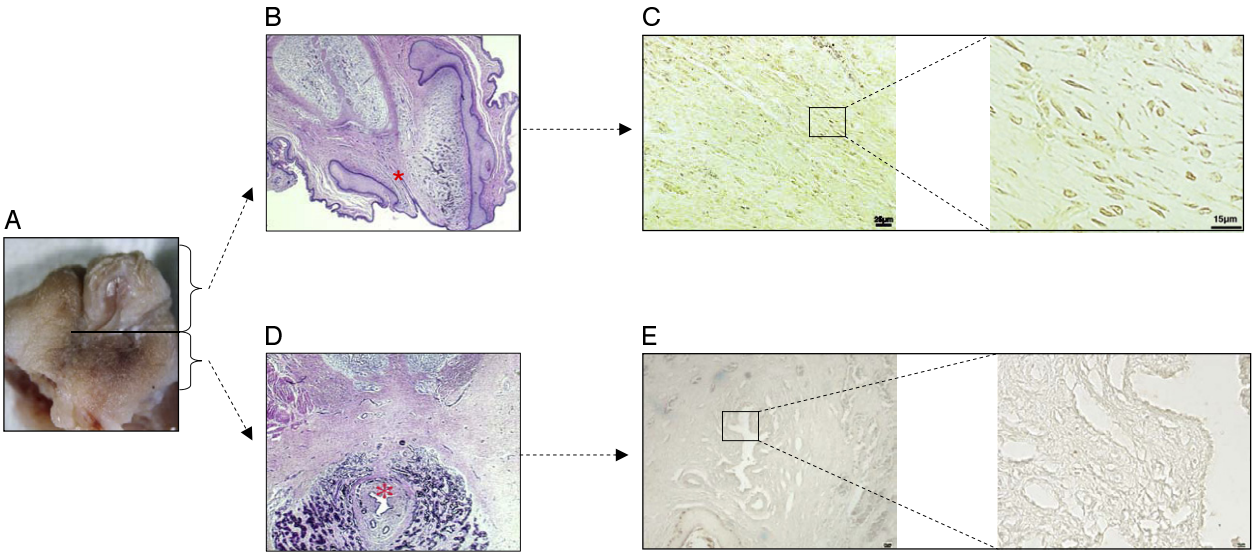

FIG. 4. *a*, macroscopic aspect of genital tubercle of hypospadiac human fetus (35 weeks of gestation). *b* and *d*, serial transverse sections of distal and abnormally open urethra (asterisks) stained with hematoxylin and eosin. Reduced from ×40. *c* and *e*, immunochemical staining for ATF3 antibody. Left column, reduced from ×100. Right column, reduced from ×400. This experiment shows this pathological part of urethra expresses ATF3 in high quantity in urethral plate, and this expression is particularly noticeable at level of ectopic urethral meatus. Serial sections of normal proximal urethra (part *e*) did not exhibit this high quantity of ATF3 protein around urethra, and were comparable to control in figure 3.

demonstrated 86% of the hypospadias samples were positive for expression of *ATF3*, whereas only 13% of those from normal tissues were positive.[16] Finally, *ATF3* expression and promoter activity in human foreskin fibroblasts are responsive to in vitro exposure to ethinyl estradiol.[16] Taken together, these results led us to confirm the implication of *ATF3* in the occurrence of hypospadias by sequencing this gene and looking for an unusual expression at the location of the ectopic meatus.

Additionally, this gene was aberrantly and specifically expressed at the location of the malformation in the human fetus. By comparison, ATF3 was expressed neither in the normal part of the urethral plate below the hypospadias nor in the urethra of the normal fetus. This expression pattern is in accordance with the previously reported hypothesis of a cell cycle regulatory effect of ATF3. Over expression of ATF3 is known to suppress cell growth, and ATF3 is also involved in the p53 dependent and independent apoptotic pathways.[17] Furthermore, over expression of ATF3 can result in slowed cell cycle progression, indicating a negative role for ATF3 in this process.[17] Normal male phallic development depends on lengthening of the urethra and fusion of the urethral folds so that the urethral opening occurs at the tip of the penis rather than more proximally.[18] Our results support the idea that abnormal expression of ATF3 somewhere on the urethral plate could alter cell cycle progression, thus creating an ectopic urethral opening at the place of ATF3 expression and impairing fusion of the distal urethral folds. It remains to be determined whether *ATF3* is the primary catalyst for the occurrence of hypospadias or if its expression is secondary to an exogen stressor, since *ATF3* is known to be a stress gene.[19,20]

The mechanisms by which the gene variants we report could be associated with hypospadias remain unknown, and the association of mutations with hyper expression could be contradictory at first glance. However, based on the positions of the variants we found and on the high quantity of ATF3 protein in hypospadias, we hypothesize an alteration of the regulation of *ATF3*. *ATF3* is indeed composed of the basic region and a leucine zipper domain from 88 to 147 amino acids, which is required for dimer formation.[15] This homodimer of ATF3 binds specifically to DNA and represses transcription from various promoters with ATF sites.[15,21]

ATF3ΔZip is a naturally occurring form of ATF3 without the leucine zipper domain resulting from alternative splicing. In contrast to ATF3, ATF3ΔZip does not form homodimers or bind DNA and, thus, prevents the repressing action of ATF3,[15] presumably by sequestering inhibitory cofactors away from the promoters.[22] Thus, ATF3ΔZip is an important element in the regulation of ATF3 function. The variants we found in patients with hypospadias are between or close to the splicing sites of *ATF3*ΔZip and may alter its expression. Thus, it is possible that these genomic defects impair the regulation process of ATF3 function and release its cell cycle suppression effect.

Two points are noteworthy. Further expression studies of *ATF3* and *ATF3*ΔZip are required to confirm this hypothesis and it is questionable why genetic variants of a widely expressed transcription factor could induce an isolated genital malformation without other apparent defects. Also, the mechanism by which the heterozygous mutation L23M induces hypospadias remains to be investigated.

CONCLUSIONS

This study of *ATF3* variants in hypospadias supports the role of *ATF3* in this frequent malformation. Functional studies of the genomic variants we describe are needed to confirm the implication of ATF3 in the occurrence of hypospadias.

Abbreviations and Acronyms

ATF3 = activating transcription factor 3
TGF-β = transforming growth factor beta

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EDITORIAL COMMENT

In extensive previous work the authors first identified the potential biological relevance of *ATF3* in hypospadias using global gene expression profiling of case and control tissues in a novel approach to candidate gene discovery. Notably, they then confirmed that *ATF3* mRNA and protein levels are increased in hypospadias, and provided further support for the potential relevance of this gene by confirming its regulation by estrogen in the fetal genital tubercle. Here, in initial sequence analysis of *ATF3*, they identify allelic variants, one a nonsynonymous polymorphism, in a subset of hypospadias cases, and based on their location they argue that these variants may plausibly affect expression of an isoform that regulates *ATF3* function. Although their genetic analysis of control samples is limited, these results mirror a larger study of *ATF3* recently published, which identifies a significant risk haplotype associated with hypospadias and rare (in 1 case potentially functional) variants in cases but not controls.[1] Unlike the present study, the expression of ATF3 protein was observed in the normal human fetus at 15 weeks of gestation but not before.

As noted by the authors, additional functional data will be needed that support a role for *ATF3* in the pathogenesis of hypospadias. However, these initial data provide a strong basis for further studies. Such studies are critical for identifying the interaction of genes and environment in the etiology of hypospadias.

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