Activating transcription factor 3: a hormone responsive gene in the etiology of hypospadias

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Abstract

Introduction: Hypospadias is a common inborn error of the genital development, whose complex etiology remains elusive. Defects of the androgen metabolism and activity have been found in a subset of boys with hypospadias. Moreover, the balance between androgens and estrogens seems to be important to the proper male genital development. Activating transcription factor 3 (ATF3), an estrogen responsive gene, has been reported to be expressed during sexual development and up-regulated in hypospadic genital skin. We investigated ATF3 as a candidate gene for hypospadias.

Material and methods: Genotyping of eight-tagged single nucleotide polymorphisms (SNP)s was performed in 330 boys with hypospadias and in 380 healthy controls. Screening for mutations in ATF3 was conducted in a subset of boys with hypospadias. ATF3 expression was evaluated in the foreskin of boys with hypospadias and in healthy controls and in the human fetal genitalia by immunohistochemistry.

Results: Three common SNPs, spanning a region of about 16 kb in intron 1 of ATF3, are associated with hypospadias. These SNPs are not linked and their effects are independent. The combination of the three risk SNPs yields the highest significance. Mutation screening identified the gene variant c536A>G in one patient and c817C>T in the 3’-UTR in two other patients. ATF3 expression was evidenced in the developing male urethra.

Conclusions: ATF3 gene variants influence the risk of hypospadias. Its hormonal responsiveness may underlie this risk effect. But also other ATF3-dependent biological aspects, such as cell survival and death, response to stress stimuli, or the control of epithelial-mesenchymal interactions, may be of importance.

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Introduction

The development of the male external genitalia is a complex process, comprising genetic programming, cell differentiation, hormonal signaling, enzyme activity, and tissue remodeling, which follows an orderly sequence, occurring in a time- and concentration-dependent way (1). Any disturbance in these processes may lead to hypospadias, a midline fusion defect of the male ventral urethra, defining a continuum, from a malformed urethral meatus at the subcoronal margin, or on the glans penis, to an intersex condition. Hypospadias affects 0.3–7 of 1000 live male births (2). Despite being so common, its etiology is largely elusive. Familial clustering of hypospadias among first-degree relatives, as well as twin studies and segregation analysis, have supported a strong heritable component in hypospadias. Genes and environmental factors are believed to influence the risk to this disorder of the male sexual development (3, 4).

Androgens and the androgen receptor are essential for the development of the male external genital (5). But defects of the androgen metabolism and activity have been found in only a small subset of patients with hypospadias. On the other hand, the importance of estrogens in male reproductive tract development has been increasingly acknowledged. Functional Estrogen Receptors (ESR)s were detected in differentiating male external genitalia localized at the same structures as the androgen receptors, indicating interactions between effects exerted by the two steroid hormones (7, 8); and an excess of estrogens in the developing murine urethra results in an inhibition of the cell proliferation in a dose-dependent manner (9). Moreover, hypospadias has been induced in a mice model by maternal exposure to synthetic estrogens during pregnancy (10). And polymorphisms in the gene for the estrogen receptor 2 have been associated with hypospadias in humans (11). It has therefore been proposed that hypospadias and other disorders of the otherwise androgen-dependent male sexual development may
occur due to the occurrence of androgen–estrogen imbalances, which can be influenced by environmental, hormonal, and genetic factors (12–14).

The up-regulation of estrogen-responsive genes has been documented in hypospadias (15, 16). From these genes, the activating transcription factor 3 (ATF3), a member of the ATF/CREB family of transcription factors (17), has been the gene with the greatest up-regulation. This gene has also been shown to be generally up-regulated during sexual differentiation (18). This evidence indicates a potential role of ATF3 in hypospadias and lead us to investigate ATF3 gene sequence in patients with this inborn error of development. We also evaluated the expression pattern of ATF3 in the human developing genitalia and in the genital skin of pre-pubertal boys with and without hypospadias.

Methods

Patients and controls

For sequence analysis, DNA from 330 boys with non-syndromic hypospadias was analyzed. As control group, we used DNA from ethnically comparable, anonymous samples, constituting 380 healthy voluntary blood donors at Karolinska University Hospital, Sweden. Genomic DNA was extracted from blood using a standard phenol/chloroform protocol.

For expression analysis, 16 human genital skin samples, from ethnically comparable boys, obtained during surgery in Stockholm, were selected: 10 pre-pubertal boys with hypospadias (6 severe and 4 mild cases) and 6 healthy boys that underwent circumcision. Human genital fetal tissue was also analyzed. The specimens, five males and five females, from the 9th to 15th weeks of gestation were obtained following elective abortion and showed no signs of maceration or macroscopic abnormality. The age of the fetuses was estimated from crown-rump length, as defined by Mull (19), using the standard tables of Moore (20) and Putten (21). The anogenital structures were removed en bloc from the fresh fetuses and fixed in formalin. The specimens were routinely embedded in paraffin, processed into 4 μm serial transverse sections, and dried at 60 °C overnight.

An ethics committee has approved the protocols for the research project. And it conforms to the provisions of the Declaration of Helsinki in 1995 (as revised in Edinburgh 2000). Subjects, and/or their parents, gave informed consent and patient anonymity was preserved. Gene nomenclature is described according to recommendations by den Dunnen & Antonarakis (22, 23).

SNP selection and typing

Eight haplotype-tagging SNPs in the ATF3 gene were selected from the genotypes of the CEPH (Centre d’Etude du Polymorphism Humain) from Utah (CEU) HapMap samples on the HapMap database (http://www.hapmap.org/) using Haplovie view version 4.0 (Broad Institute of MIT and Harvard, MA, US) (25) with a logarithm of odds (LOD) cut-off of 3.0 and an $r^2 > 0.8$, including only SNPs with a minor allele frequency of 2% (Table 1). SNP typing was performed using a 5’-nuclease TaqMan assay together with fluorescently labeled probes using standard protocols (Applied BioSystems, Warrington, UK). The samples were analyzed on an ABI 7900HT.

Genetic statistics

To identify potentially associated polymorphisms, we performed general tests of allele ($2 \times 2$ contingency table and a Pearson test with 1-df) and genotype ($2 \times 3$ contingency table with a Pearson test with 2-df) frequency differences among the cases and the controls. Odd ratios (ORs) and OR 95% confidence intervals were estimated by the Woolf method (24). These analyses were conducted using Statistica 7.0. Further, single-locus and multi-marker haplotype association tests were performed using two different algorithms, Haplovie view version 3.32 (25) and Unphased version 3.0.10 (26). The calculations were performed allowing for missing data estimation. The threshold for rare haplotype frequency identification was 0.005. The $P$ values were corrected for multiple testing by performing permutations as follows: the trait values were randomly shuffled between subjects 1000 times. In each permutation, the minimum $P$ value is compared with the minimum $P$ value observed in all the analyses in the original dataset. Pair-wise linkage disequilibrium (LD) between polymorphisms was measured by estimating $D^\prime$ (the normalized disequilibrium coefficient that ranges from 0 to 1); and the squared correlation coefficient $r^2$. Hardy–Weinberg equilibrium of the genotype frequencies at each SNP in controls and cases was accessed with Haplovie view version 3.32 (25).

To determine whether the associated risk gene variants were independently associated, logistic regression analysis was performed using the SAS computer program (SAS Institute Inc., Cary, NC, USA) where the proc logistic command was used. This resulted in a standard logistic regression model in which the regression coefficients were logarithms of the ORs. The models were compared using the log-likelihood ratio test that approximates to a $\chi^2$

<table>
<thead>
<tr>
<th>No.</th>
<th>Marker</th>
<th>NCBI position</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>rs2137424</td>
<td>6268496</td>
</tr>
<tr>
<td>2</td>
<td>rs3125289</td>
<td>6256926</td>
</tr>
<tr>
<td>3</td>
<td>rs1877474</td>
<td>6275670</td>
</tr>
<tr>
<td>4</td>
<td>rs11119982</td>
<td>6282623</td>
</tr>
<tr>
<td>5</td>
<td>rs10735510</td>
<td>6303239</td>
</tr>
<tr>
<td>6</td>
<td>rs9429689</td>
<td>6307308</td>
</tr>
<tr>
<td>7</td>
<td>rs12070345</td>
<td>6308114</td>
</tr>
<tr>
<td>8</td>
<td>rs10479</td>
<td>6311392</td>
</tr>
</tbody>
</table>
distribution with 1 df. Initially, the different genetic models for each marker against each other were tested. The analysis presented in Table 6 only includes data for the best model. In order to get OR estimates for the different genotypes, variables were recoded into design variables.

**PCR and sequencing**

DNA from 102 patients was selected for sequencing. This group consisted of 93 boys with non-syndromic hypospadias, 69 Swedish and 24 non-Swedish patients, mostly of Middle Eastern origin: 47 presented a moderate-to-severe phenotype, while the remaining patients have a mild form of hypospadias, and 33 were familial and 60 were sporadic cases. The remaining nine patients presented hypospadias and anal atresia at birth and are of Swedish ethnicity. As control group for this screening, we used 96 of the aforementioned controls. Genomic sequences were obtained from the National Centre for Biotechnology Information (NCBI) and confirmed at Ensembl Genome Browser. Primers flanking the exon/intron junctions were designed by the Primer 3 (http://primer3.sourceforge.net/) program and used to amplify the four exons and exonic–intronic borders, 5′- and 3′-UTR regions, and the putative promoter region of the ATF3 gene (Table 2). PCRs were performed with DyNAzyme EXT DNA Polymerase (Finnzyme, Espoo, Finland) following the manufacturer protocols. After ExoSap-IT enzyme (USB Europe GmbH, Staufen, Germany) treatment, the PCR fragments were performed with DyNAzyme EXT DNA Polymerase (Finnzyme, Espoo, Finland) treatment, the PCR fragments were sequenced on both direction using BigDye Terminator protocols. After ExoSap-IT enzyme (USB Europe GmbH, Staufen, Germany) treatment, the PCR fragments were sequenced on both direction using BigDye Terminator v3.1 kit (Applied BioSystems) and analyzed in ABI Prism 3730 Sequencer. Sequence analysis was performed in the program SeqScape v2.5 (Applied BioSystems).

**Homology and structure predictions**

The putative homologs of the human ATF3 were detected by HomoloGene at http://www.ncbi.nlm.nih.gov/. The alignments were made with ClustalW (European Bioinformatics Institute (EBI), European Molecular Biology Laboratory (EMBL), Cambridge, UK). Secondary structures were predicted with NNPREDICT, University of California (http://alexander.compbio.ucsf.edu/~nomi/nnpredict.html). Three-dimensional structure was predicted at Protein Homology/analogY Recognition Engine (http://www.sbg.bio.ic.ac.uk/~phyre) from the Structural Bioinformatics Group, Imperial College, London, UK.

**Immunohistochemistry**

The rabbit antibody anti-ATF3 (Santa Cruz Biotechnology, California, US) and the secondary anti-rabbit antibody (Vector Laboratories Ltd, California, US) were obtained commercially. The tissues were fixed for 5–7 h in 4% formalin, washed four times for 10 min in PBS, and placed thereafter in 70% EtOH before paraffin embedding. After dewaxing and rehydration, the paraffin sections were treated for antigen retrieval by heating at 98 °C in 0.1 M Tris (pH 9.0) for 20 min after which the slides were allowed to cool to room temperature. The sections were then treated with 1 M H2O2 for 15 min in a dark-blocked endogenous peroxidase activity. To block non-specific antibody binding, sections were pre-incubated in 10 mg/ml BSA (Sigma) containing 10% goat non-immune serum (Vector) for 40 min. Affinity-purified polyclonal anti-ATF3 was applied to the sections at a 1:800 dilution in 10 mg/ml BSA and allowed to incubate at 4 °C overnight in a humid chamber. The sections were incubated with the biotinylated secondary goat anti-rabbit antibody (Vector Laboratories Ltd.) diluted in buffer containing 1% BSA and 10% goat serum followed by enzyme conjugate application (avidin-biotin complex, Vector Laboratories Ltd.) and chromogen development (antibody-enzyme conjugate, Vector Laboratories Ltd.). All sections were counterstained with hematoxylin (Merck) for 15 s before mounting in Kaiser’s glycerin gelatin (Merck). Images of immunostained tissues were captured using a Zeiss microscope. Background controls were performed similarly using only the secondary antibody.

**Results**

**Genotyping**

Genotyping of the eight haplotype-tagging SNPs yielded a success rate of more than 95%. All loci were in Hardy–Weinberg equilibrium. Single-locus analysis of the allele and genotypes frequencies of patients versus controls indicates that three of the studied polymorphisms in ATF3 are associated with hypospadias: the T allele and the TT genotype in rs3125289; the T allele and the TT genotype in rs1877474; and the C allele and the CC genotype in the rs11119982. The estimated P value after correcting for multiple comparisons by performing 1000 permutations remains below 0.05 only for the
SNP rs11119982 (Tables 3 and 4). In spite of that, we pursued the analysis of the three SNPs.

The study of haplotype effects, allowing for missing data such as uncertain phase and missing genotypes, has identified that the combination of the three risk alleles in the SNPs rs3125289 (T), rs1877474 (T), and rs11119982 (C) actually yields the higher effects when compared with the analysis of the alleles individually or when combined two-by-two; and the combination of TTC is the most strongly associated with hypospadias in our study (Table 5). The estimated \( P \) value after correcting for multiple comparisons by performing 1000 permutations remains significant. The \( D' \), the normalized disequilibrium coefficient, is very low (0.06) evidencing that the alleles are not in LD. Other marker combinations were not significantly associated with hypospadias.

In order to test if the risk effect of the three risk alleles in the SNPs rs3125289 (T), rs1877474 (T), and rs11119982 (C) were independently associated with hypospadias, we used logistic regression analysis. As the \( P \) value for the presence of the risk alleles was the lowest, we used co-dominant coding when comparing with the fit of models containing the three risk markers. Three models were tested, one with only one polymorphism, one with two polymorphisms, and one with the three polymorphisms together. All the tested models yielded significant \( P \) values. The model containing the three risk alleles performs better (lowest \( P \) value) than the other two models (Table 6). However, the test including interaction terms did not perform better than the previous test, suggesting that these three gene variants are not in epistasis, and their conjoined effect may be only additive.

The control population constituted of healthy blood donors in Stockholm, which includes individuals from this ethnicity. However, we cannot access in detail this population, since it is totally anonymous, which could represent a bias to our study. However, we controlled this problem by analyzing separately the different ethnic groups within the patient groups. And the results are independent of the ethnical origin. The allele frequencies are similar in the different groups; \( \chi^2 \) test to access differences in frequencies yielded non-significant \( P \) values (data not shown).

Mutation screening

Mutation screening performed in 93 patients and 96 controls revealed several genetic variants (Fig. 1). A missense mutation in exon 3, c536A>G, causing an amino acid substitution, Arg90Gly, was found in one Swedish patient with a sporadic moderate form of hypospadias. An 3'-UTR gene variant, c817C>T, was found in two patients, one Swedish patient with a sporadic form of hypospadias and one boy from Middle Eastern with a familiar form, both moderate-to-severe phenotypes. The three affected boys are heterozygous for the risk alleles in rs1877474 and rs11119982. These newly identified gene variants were not found in the control population.

### Table 3

Cases (Ca) versus controls (Co) study for single-locus analysis: three of the studied polymorphisms in activating transcription factor 3 (ATF3) are associated with hypospadias: rs3125289, rs1877474, and rs11119982.

<table>
<thead>
<tr>
<th>SNP</th>
<th>Genotype</th>
<th>Case</th>
<th>Control</th>
<th>Ca-Freq</th>
<th>Co-Freq</th>
<th>( P ) value</th>
<th>OR (95% CI)</th>
</tr>
</thead>
<tbody>
<tr>
<td>rs2137424</td>
<td>CC</td>
<td>34</td>
<td>47</td>
<td>0.1164</td>
<td>0.1288</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>CT</td>
<td>124</td>
<td>152</td>
<td>0.4247</td>
<td>0.4164</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>TT</td>
<td>134</td>
<td>166</td>
<td>0.4589</td>
<td>0.4548</td>
<td>0.8901</td>
<td>NS</td>
</tr>
<tr>
<td>rs3125289</td>
<td>CC</td>
<td>53</td>
<td>101</td>
<td>0.2181</td>
<td>0.2708</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>CT</td>
<td>113</td>
<td>189</td>
<td>0.465</td>
<td>0.5067</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>TT</td>
<td>77</td>
<td>83</td>
<td>0.3169</td>
<td>0.2225</td>
<td>0.02831</td>
<td>TT: 1.62 (1.13–2.33)</td>
</tr>
<tr>
<td>rs1877474</td>
<td>CC</td>
<td>46</td>
<td>81</td>
<td>0.1365</td>
<td>0.2166</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>CT</td>
<td>157</td>
<td>171</td>
<td>0.4659</td>
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</tr>
<tr>
<td></td>
<td>TT</td>
<td>134</td>
<td>122</td>
<td>0.3976</td>
<td>0.3262</td>
<td>0.01108</td>
<td>TT: 1.36 (1.01–1.85)</td>
</tr>
<tr>
<td>rs11119982</td>
<td>CC</td>
<td>82</td>
<td>60</td>
<td>0.2485</td>
<td>0.1604</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>CT</td>
<td>160</td>
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<td>0.4848</td>
<td>0.4492</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>TT</td>
<td>88</td>
<td>146</td>
<td>0.2667</td>
<td>0.3904</td>
<td>4 × 10^{-4}</td>
<td>CC: 1.73 (1.19–2.51)</td>
</tr>
<tr>
<td>rs10735510</td>
<td>AA</td>
<td>91</td>
<td>127</td>
<td>0.2809</td>
<td>0.3096</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>AC</td>
<td>158</td>
<td>179</td>
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</tr>
<tr>
<td></td>
<td>CC</td>
<td>75</td>
<td>68</td>
<td>0.2315</td>
<td>0.1818</td>
<td>0.1327</td>
<td>NS</td>
</tr>
<tr>
<td>rs9429889</td>
<td>CC</td>
<td>235</td>
<td>285</td>
<td>0.7015</td>
<td>0.756</td>
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</tr>
<tr>
<td></td>
<td>CT</td>
<td>90</td>
<td>86</td>
<td>0.2687</td>
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<tr>
<td>rs12070345</td>
<td>AA</td>
<td>79</td>
<td>75</td>
<td>0.2365</td>
<td>0.206</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>AG</td>
<td>170</td>
<td>174</td>
<td>0.509</td>
<td>0.478</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>GG</td>
<td>85</td>
<td>115</td>
<td>0.2545</td>
<td>0.3159</td>
<td>0.1847</td>
<td>NS</td>
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<tr>
<td>rs10475</td>
<td>CC</td>
<td>221</td>
<td>252</td>
<td>0.6637</td>
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</tr>
<tr>
<td></td>
<td>CT</td>
<td>96</td>
<td>116</td>
<td>0.2833</td>
<td>0.3061</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>TT</td>
<td>16</td>
<td>11</td>
<td>0.04805</td>
<td>0.02902</td>
<td>0.3906</td>
<td>NS</td>
</tr>
</tbody>
</table>

Empirical 5% quantile of the best \( P \) value after 1000 permutations: 0.007799 (Unphased version 3.0.10). Significant results are in bold.

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Homology analysis of the *ATF3* mRNA revealed that the c536A>G gene variant is located in a highly conserved locus (Fig. 2). Furthermore, protein structure prediction suggests that this variant is predicted to induce a change in the protein structure (Fig. 3; detail in Fig. 4). This evidence indicates that the variant c536A>G is likely to have a functional consequence.

Analysis of the c817C>T locus has shown that it is not conserved (Fig. 2). However, it is predicted to be a highly regulatory sequence (data not shown). A new polymorphism was found in the promoter region, g.210848306delC, in heterozygous form in 7 out of 93 patients and 8 out of 96 controls. This polymorphism is not associated with hypospadias (*P* > 0.3).

**Immunohistochemistry**

**ATF3 protein expression in human prepubertal samples**  
*ATF3* protein expression is observed at the nuclei of stromal cells and the vascular endothelium in foreskin tissue samples from prepubertal boys with hypospadias and in healthy controls. The number of positive cells or the staining intensity did not differ notably between the patient group and the controls (Fig. 5). In a few samples from patients and controls, some positively stained cells could be observed in the epidermal region. The *ATF3* staining was then seen in both nuclei and cytoplasm in these cells of different origins (Fig. 6).

**ATF3 protein expression in human fetal samples**  
Human fetal sections from gestational ages 9–15 weeks were analyzed for *ATF3* expression by immunohistochemistry. *ATF3* expression is seen in the urethral wall at 15th week of gestation particularly in the nucleus of epithelium cells, preferentially adjacent to the nuclear membrane (Fig. 7). A similar pattern of expression was observed in the developing vagina at every observed stage. Nuclear *ATF3* expression was observed consistently in the rectum walls in both male and female fetuses.

**Table 5** Study of haplotype effects, allowing for missing data such as uncertain phase and missing genotypes.

<table>
<thead>
<tr>
<th>Haplotype</th>
<th>Case</th>
<th>Control</th>
<th>Ca-Freq</th>
<th>Co-Freq</th>
<th>χ²</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>C–C–C</td>
<td>70.1</td>
<td>52.34</td>
<td>0.1028</td>
<td>0.06906</td>
<td>0.4544</td>
<td>0.5003</td>
</tr>
<tr>
<td>C–C–T</td>
<td>49.66</td>
<td>87.4</td>
<td>0.07282</td>
<td>0.1153</td>
<td>15.21</td>
<td>9.64 × 10⁻²³</td>
</tr>
<tr>
<td>T–C–C</td>
<td>113.2</td>
<td>170.5</td>
<td>0.166</td>
<td>0.2249</td>
<td>7.957</td>
<td>0.004789</td>
</tr>
<tr>
<td>T–C–T</td>
<td>94.9</td>
<td>129.6</td>
<td>0.1392</td>
<td>0.171</td>
<td>0.03103</td>
<td>0.8602</td>
</tr>
<tr>
<td>T–T–C</td>
<td>37.52</td>
<td>68.89</td>
<td>0.05501</td>
<td>0.09088</td>
<td>11.95</td>
<td>5.5 × 10⁻²</td>
</tr>
<tr>
<td>T–T–T</td>
<td>96.99</td>
<td>22.99</td>
<td>0.1422</td>
<td>0.03033</td>
<td>56.89</td>
<td>4.6 × 10⁻¹⁰¹</td>
</tr>
</tbody>
</table>

The reference haplotype was CTT, the most common haplotype in the control population. The combinations of the three risk alleles in the *ATF3* SNPs rs3125289 (T), rs1877474 (T), and rs11119982 (C) yield the higher effects in our study. The empirical 5% quantile of the best *P* value is 0.002013 after 1000 permutations. *D* = 0.06. Ca, case; Co, control.

*Putative protective haplotype.*  
*Putative susceptibility haplotype.*  
Significant results are in bold.

The estimated *P* value after correcting for multiple comparisons by performing 1000 permutations* remains below 0.05 for rs1877474 and rs11119982. Permutation *P* value calculated with Unphased and Haploview softwares allowing for missing data such as uncertain phase and missing genotypes. The three affected boys where the mutations 536A>G and c817C>T were found are heterozygous for the risk alleles in rs1877474 and rs11119982.
We report three SNPs in the $ATF3$ sequence, spanning a region of about 16 kb in intron 1, which affect the risk for hypospadias. These risk SNPs are not linked and show independent association with hypospadias, as we did not find evidence of epistasis. However, the presence of all three markers yields the most significant result, which indicates an additive effect; it may also suggest that these markers mark a so far unidentified risk increasing gene variant. Additionally, a missense mutation in exon 3 and a sequence variant in the 3'-UTR region of this gene are present in three non-related boys with hypospadias. The first
causes an amino acid change that seems to be responsible for a change in the protein structure. The variant located in the 3'-UTR region may affect regulatory events.

We also showed that ATF3 is expressed in the human developing male urethra and in the genital skin of prepubertal boys. This evidence strengthens previous reports implicating ATF3 in genital development and hypospadias.
Figure 4 Detail of the protein prediction to ATF3 and ATF3 carrying the mutations 536A > G (ATF3 mut). Predicted at PHYRE–Structural Bioinformatics Group Imperial College, London.

Figure 5 Immunohistochemistry results with ATF3 expression in genital skin tissue. ATF3 expression by immunohistochemistry in human prepubertal genital skin from patients and control individuals. ATF3 expression is localized predominantly in the nuclei in hypospadias patients and healthy controls. Staining in all control and hypospadias individuals was similar, localizing in the nuclei with some predominance around the nuclear membrane in the stromal region of the foreskin (arrows). (A) A representative section of a genital skin tissue from a healthy boy showing positive staining for ATF3 localizing in the nuclei and around the nuclear membrane in the stromal cells (arrowheads) and in the vascular endothelium of the foreskin (arrow). All genital skin tissues from control individuals and hypospadias patients at different ages showed similar staining. (B) The background control using only the secondary antibody (sections photographed at 400 × magnification).
associates \textit{ATF3} sequence variants with an increased risk to hypospadias. The mechanisms that explain how variants in the sequence of \textit{ATF3} affect the risk to hypospadias are not yet known; but some hypotheses can be suggested.

Variants in \textit{ATF3}, a gene that is up-regulated in hypospadias, may affect the risk to this hormone-dependent disorder. This effect may be due to disturbances on estrogenic responses during development (15, 16, 18). Moreover, \textit{ATF3} expression has also been reported to be up-regulated by the androgen receptor’s activation by both androgens and estrogens in cell lines (27); this aspect indicate that variants in \textit{ATF3} may underlie androgen–estrogen imbalances and influence the responsiveness to endocrine disrupters. Furthermore, \textit{ATF3} is a key immediate early gene induced by gonadotropin-releasing hormone (GnRH) \textit{in vivo} and \textit{in vitro} (28). The GnRH is a key regulator of mammalian reproduction and is required for synthesis and secretion of luteinizing hormone (LH) and follicle-stimulating hormone (FSH), two essential signals in the establishment of primary and secondary sexual characteristics. Interestingly, LH and FSH response to GnRH is higher in boys with hypospadias than those in prepubertal control subjects (29); and genetic variants of the LH and its receptor have been associated with disorders of the male reproductive tract (30, 31).

Not only endocrine regulation but also other \textit{ATF3}’s-dependent biological aspects may be disturbed by gene

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{fig6.png}
\caption{\textbf{Figure 6} \textit{ATF3} expression by immunohistochemistry in human prepubertal genital skin from a boy with severe hypospadias. In this specific patient with severe hypospadias (A and B), \textit{ATF3} expression is observed in both dermal and epidermal cells. The staining is mostly nuclear, but in certain epidermal cells of different origins, \textit{ATF3} expression (arrows) is localized in the cytoplasm (sections photographed at 400× magnification).}
\end{figure}

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{fig7.png}
\caption{\textbf{Figure 7} \textit{ATF3} expression by immunohistochemistry in posterior urethra from fetal gestation week 15. (A) \textit{ATF3} expression is seen in urethral wall, particularly in the epithelium, localizing in the nucleus preferentially located adjacent to the nuclear membrane; no expression was observed before the 15th week. Similar expression pattern is seen in female vagina from 9th to 15th gestational weeks. (B) A similar section of the developing male urethra at 11 weeks shows no \textit{ATF3} immunostaining (sections photographed at 400× magnification).}
\end{figure}
variants in this gene. Indeed, ATF3 is normally expressed at a steady state in quiescent cells. It has traditionally been related to cell survival and/or apoptosis, stress, DNA damage, homeostasis, wound healing, cell adhesion, inflammation, nutrient limitation, and chemical exposure (32–36). Moreover, overexpression of ATF3 suppresses cell growth, by slowing the progression of cells from G1 to S phase, and it has been related to pro-apoptotic events and in the regulation of cell fate (36, 37). This is a relevant aspect due to the importance of cell proliferation and apoptosis regulation during development (38, 39).

Furthermore, ATF3 responds to signals in epithelial cells via the transforming growth factor-β (TGF-β) pathway (39, 40). Interestingly, genes involved in the TGF-β pathway that facilitates epithelial–mesenchymal interactions are up-regulated during several stages of urethral tube development in mice (12, 40–42). Moreover, ATF3 lies at the centre of interactions between the TGF-β signaling pathway and steroid hormone receptors (12). Indeed, the normal male genital tubercle development depends on well-coordinated epithelial–mesenchymal interactions (2).

ATF3 seems to be involved in crucial aspects of urethral and genital developments. It is possible that variants in the ATF3 sequence and/or environmental mechanisms, such as endocrine disrupters (9, 10, 12) and lower nutrient availability (35) (a link to low birth weight? (4)) lead to inappropriate regulation of ATF3, which might halter the seam fusion process of the developing urethra, by suppressing cell growth and/or promoting cell death, by disturbing hormonal signaling, or by impairing epithelial–mesenchymal differentiation, and result in a shorter urethra with a proximal opening.

In summary, the evaluation of ATF3 as a candidate for hypospadias indicates that sequence variants of this gene, which is expressed in the human developing male urethra and may play a central role in urethral development, may be involved in the genetic risk for hypospadias in different ways: by the effect of three common low risk SNPs in the intron 1 of ATF3; or induced by less common variants, possibly with higher effect, such as the missense mutation c.536A>G in exon 3 and c.817C>T in the 3′-UTR.

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