Evidence for Epigenetic Abnormalities of the Androgen Receptor Gene in Foreskin from Children with Hypospadias

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Context: Hypospadias is a malformation of the penis due to an incomplete development of the male urethra, the exact etiology of which in the majority of cases remains unknown.

Objective: The objective of the study was to assess whether defects of the androgen receptor (*AR*) gene (CAG repeats and methylation pattern) and DNA methyltransferases (*DNMT*) family are present in hypospadic patients.

Design: CAG repeats length, methylation status, and expression of the *AR* gene were analyzed. The *DNMT* family was studied at the protein level and the *DNMT3A* sequenced.

Setting: The study was performed at a pediatric endocrinology referral clinic.

Patients or Other Participants: Twenty boys with isolated glandular hypospadias and 20 age-matched control children undergoing a surgical procedure for circumcision were studied.

Main Outcome Measure(s): CAG repeats length and AR methylation pattern in PBLs and foreskin tissue, *DNMT* expression and sequencing in patients and controls, and *in vitro* studies in cultured fibroblasts were measured.

Results: AR gene methylation in foreskin tissues from patients with hypospadias was higher than in normal children. *AR* expression in foreskin tissue of hypospadic patients was lower than in controls, whereas the *DNMT3A* protein level was significantly higher in patients than controls. In cultured fibroblasts, both dihydrotestosterone and testosterone significantly reduced *AR* gene methylation and *DNMT3A* expression in a dose-dependent fashion and increased *AR* expression.

Conclusion: The *AR* gene in target tissues from patients with hypospadias is more methylated than in control children, resulting in a decreased expression of the *AR*. The mechanism underlying the modulation of the *AR* gene expression seems to be mediated by *DNMT3A*. This epigenetic alteration of the *AR* gene might be involved in the pathogenesis of hypospadias. **(*J Clin Endocrinol Metab* 96: E1953–E1962, 2011)**

Hypospadias is defined as a malformation of the penis due to an incomplete development of the male urethra leading to the localization of the urinary meatus on the ventral size of the penis in a variable position from the glans to the perineum (1, 2). Hypospadias affects approximately one in 250 live male births (3), but recent reports suggest an increasing incidence of hypospadias in Europe and North America (4). The exact etiology remains unknown in the majority of cases, although hypospadias has been associated with some genetic syndromes or abnor-

ISSN Print 0021-972X ISSN Online 1945-7197
Printed in U.S.A.

doi: 10.1210/jc.2011-0511 Received February 25, 2011. Accepted August 24, 2011.
First Published Online September 20, 2011

Abbreviations: AR, Androgen receptor; DHT, dihydrotestosterone; DNMT, DNA methyltransferase; M, methylated; PBL, peripheral blood leukocyte; T, testosterone.

malities in the androgen receptor (AR) (2, 5). Several candidate genes and their response to environmental agents have been analyzed both in human and mouse tissues, opening many promising areas of research in relation to the etiology of hypospadias (6). Among these genes, the activating transcription factor 3 and the *TGF-β* resulted to be up-regulated in human and mouse hypospadic tissues compared with controls the former and during sex differential development in the mouse genital tubercle the latter. In addition to these genes, recently a new gene called *MAMLD1* (mastermind-like domain containing 1) (previously known as chromosome X open reading frame 6) has been associated with hypospadias, further indicating that hypospadias is a highly heterogeneous condition subject to the influence of multiple genetic and environmental factors (7).

Normal male phallic urethral development is regulated by testosterone (T) produced by the fetal testis and transformed peripherically into dihydrotestosterone (DHT), which binds to the AR (8). Thus, any event leading to an insufficient androgen action in the male fetus may result in undervirilization in the newborn (9).

The *AR* gene (NG_009014.1) contains an approximately 1.5-kb region extending across the transcription start sites that is 65% G+C with an observed/expected CpG ratio of 0.62 (so-called dinucleotide CpG) (10). CpG sites are not randomly distributed in the genome; instead, there are CpG-rich regions known as CpG islands, which span the 5′ end of the regulatory region of many genes (11). In humans, CpG islands are important because they are sites of DNA methylation, which in turn, has a critical role in the control of gene expression and activity, genomic imprinting, and X chromosome inactivation (11, 12). DNA methylation is catalyzed mainly by three DNA methyltransferases, DNMT1, DNMT3A, and DNMT3B (13). DNMT1 has a high preference for hemimethylated DNA and is essential for maintaining methylation patterns during DNA replication (14, 15). DNMT3A and DNMT3B have a preference for both unmethylated and hemimethylated DNA and are responsible for *de novo* methylation during early development (16, 17). Furthermore, according to *in vivo* studies, DNMT3A and DNMT3B have different target sites for DNA methylation, depending on the cell types and the stage of development leading to distinct phenotypes and developmental defects in mammals (18, 19).

Several studies found links between DNA methylation and gene expression (13, 14, 20). Sequences near silent genes generally are methylated, whereas those near active regions are not. Hypermethylation of the CpG islands in the promoter region of steroid receptor genes has been associated with the transcriptional inactivation of genes and viewed as functionally equivalent to an inactivating mutation (15). The majority of studies on DNA methylation of the *AR* gene have been performed on tumoral prostatic tissues (21–23), except for two studies previously performed by our group on hirsute patients (24) and pubic hair from children with premature pubarche, implicating as a potential pathogenetic mechanism of these two conditions (20) a reduced *AR* gene methylation leading to tissue hypersensitivity to androgens. To our knowledge, no data on the association between *AR* gene methylation and genital malformation, including both hypospadias and cryptorchidism, are available.

To assess whether defects in the AR function and its regulatory mechanisms contribute to the development of hypospadias, CAG length and *AR* gene expression and methylation were analyzed in peripheral blood leukocytes (PBLs) and foreskin tissues from patients with isolated glandular hypospadias and a group of age-matched children undergoing surgical procedure for circumcision. DNMT protein levels and DNMT3A sequences were also studied to evaluate their specific role in the methylation process.

Materials and Methods

Patients and normal subjects

Twenty boys with isolated glandular hypospadias (aged 3.3 ± 1.9 yr, mean ± SD; range 1.3–6.5 yr) and 20 age-matched (3.6 ± 1.4 yr, mean ± SD; range 1.3–5.8; $P = 0.64$) control children undergoing surgical procedure for circumcision were studied. All subjects included in the study were Caucasian of Italian origin, and had a birth weight appropriate for gestational age, according to national neonatal standards (25). We excluded hypospadic patients with other malformations of genitalia or syndromes. No significant clinical data, including cryptorchidism, were recorded at first visit (Table 1). No relevant information on parents' job, places in which patients lived, and contamination by

TABLE 1. Clinical characteristics of hypospadic patients and control children

	Hypospadic patients (mean ± SD)	Control children (mean ± SD)
Age (yr)	3.3 ± 1.9	3.6 ± 1.4
Height (cm)	93.9 ± 14.2	97.6 ± 11.5
Weight (kg)	14.3 ± 3.7	15.5 ± 3.3
Birth length (cm)	49.8 ± 1.7	50.3 ± 2.1
Birth weight (kg)	3.4 ± 0.5	3.5 ± 0.4
BMI (kg/m^2)	16.1 ± 0.9	16.2 ± 1.1
Pubertal stage (Tanner)	I TV < 3 ml	I TV < 3 ml
Micropenis	0%	0%
Cryptorchidism	0%	0%
Other malformations or syndromes	0%	0%

BMI, Body mass index; TV, testicular volume.

endocrine disrupters were collected. A blood sample and foreskin tissue were obtained from all subjects. This study was approved by the Clinical Research Committee of the Department of Pediatrics at the University of Parma (protocol 18/04/2008-0012346-U-2/1/3, Parma, Italy), and informed consent was obtained from the children's parents.

Hormone assays

Baseline DHT serum concentrations were measured in all subjects. An aliquot of blood was centrifuged immediately after collection, and plasma stored at −20 C until assayed. Commercial kits were used for the measurement of serum DHT (ELISA; Diagnostic Systems Laboratories, Webster, TX). Mean intra- and interassay coefficients of variation were 6.9 and 8.5%. The sensitivity of the DHT assay was 6.0 pg/ml (0.02 nmol/liter).

Genomic DNA extraction

Genomic DNA was extracted from PBLs and foreskin tissues obtained from a total of 40 children, using a rapid extraction protocol (QIAamp kit; QIAGEN, Chatsworth, CA), according to the instructions provided by the manufacturer.

Microsatellite size determination: CAG repeats

Genomic DNA obtained from PBLs and foreskin tissue was analyzed by direct sequencing, using the sense (5′-GTG CGC GAA GTG ATC CAG AA-3′) and antisense (5′-TCT GGG ACG CAA CCT CTC TC-3′) specific primers. Amplification conditions consisted of an initial denaturing step at 95 C for 5 min, followed by 40 cycles at 95 C for 1 min, at 57 C for 1 min, and at 72 C for 1 min. Extension was carried out at 72 C for 5 min. The PCR amplified samples were analyzed by the ABI 310 genetic analyzer (Applied Biosystems, Foster City, CA).

AR gene expression

Total RNA was extracted from foreskin tissues, using the single-step liquid phase separation (TRIzol reagent kit; Molecular Research Center, Cincinnati, OH). cDNA synthesis was accomplished with the random primer using the RT-PCR kit (Roche Molecular Biochemicals, Mannheim, Germany). Real-time quantitative PCR was used for RNA quantitation. Specific primers and a fluorescent probe for androgen receptor were used (AR forward, 5′-AAG GCT ATG AAT GTC AGC CCA-3′; AR reverse, 5′-CAT TGA GGC TAG AGA GCA AGG C-3′; AR probe, FAM 5′-TGT GTG CTG GAC ACG ACA ACA ACC-3′, TAMRA). The TaqMan rRNA control primers and probe were used to detect the 18S rRNA levels that provide an endogenous control for PCR quantitative studies (TaqMan rRNA control reagents, VIC probe; Applied Biosystems). Following the TaqMan protocol, the reactions were prepared in triplicate using 25 ng of each cDNA and adding the following reagents at the following concentrations: 1× TaqMan universal PCR master mix, 900 nM of primers, 100 nM of probe, and 50 nM of 18S rRNA primers and probe. Reactions were started at 95 C for 10 min to activate AmpliTaq Gold DNA polymerase and run for 50 cycles at 95 C for 15 sec and 60 C for 1 min. PCR amplification was performed using the ABI PRISM 7700 sequence detection system (PerkinElmer Applied Biosystems). Results were normalized using the 18S rRNA. The relative abundance of *AR* gene in hypospadic patients was calculated in comparison with the controls.

AR gene methylation analysis

Bisulfite modification

DNA (1 μg) from foreskin tissues was used for the bisulfite modification as previously described (20). We have confirmed the presence of four CpG islands within the region of the AR transcription site (GenBank accession no. X78592.1) by MethPrimer (26). This domain contains a dense CG dinucleotide content that fulfills a number of strict criteria for CpG islands (10, 27). It has been shown that, in other genes, methylation of similar CpG islands either blocks gene transcription ore creates chromatin changes that are incompatible with transcription (10, 28).

Real-time quantitative methylation-specific PCR

Methylation analysis was performed by real-time PCR after sodium bisulfite treatment because this technique is suited to highly sensitive and specific detection of DNA methylation (29). We have studied the CpG island extending from −792 to −531 in the promoter region of the *AR* gene with two different fluorogenic probes recognizing the bisulfite-converted methylated (M) and unmethylated DNA, were used, respectively (20). Amplification data were collected by the ABI PRISM 7700 sequence detection system, and the methylation index was calculated (methylation index = [M/(M + unmethylated)] × 100%).

DNMT3A gene sequencing

Genomic DNA extracted from PBLs and foreskin tissue was used. *DNMT3A* gene exons (GenBank accession no. NM_175629) were amplified by PCR with intron spanning primers (Supplemental Table 1, published on The Endocrine Society's Journals Online web site at http://jcem.endojournals.org). After 5 min of denaturation at 95 C, 35 cycles of PCR amplification were performed. The general conditions used were as follows: a denaturation step of 1 min at 95 C, a hybridization step specific for each primer pair used, and an extension step of 1 min

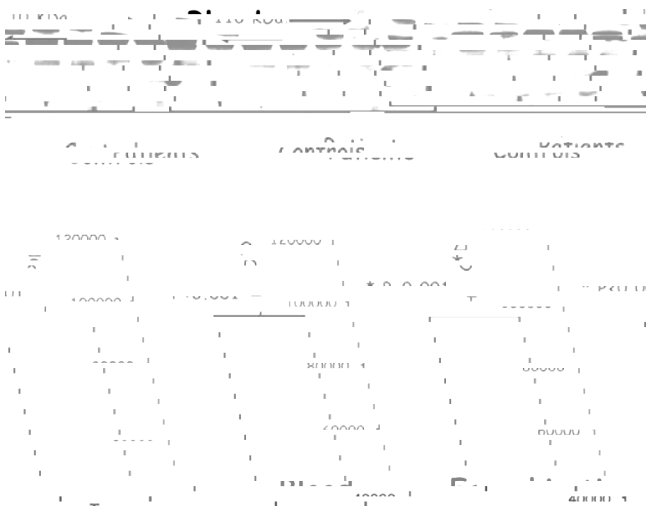

FIG. 1. CAG repeats in the *AR* gene of PBLs and foreskin tissue from patients with hypospadias and normal children. The CAG repeat length in PBLs and foreskin tissue is not the same because the number of samples analyzed is different due to the insufficient amount of DNA available.

at 72 C. All PCR fragments were purified by Biomeck (Beckman Coulter Inc., Miami, FL). Genomic DNA was then sequenced using a CEQ dye-terminator cycle sequencing kit (Beckman Coulter) according to the manufacturer's protocol. Sequence alignments were performed with the DNAStar program (Beckman Coulter). DNA sequence variations were confirmed by a separately made DNA extraction, PCR amplification, and sequence reaction. All the sequencing reactions were performed using the CEQ XL2000 DNA analysis system (Beckman Coulter).

Genotype frequencies of detected polymorphisms were compared with data of International HapMap Project (http://www.hapmap.org/), using the CEU population. The numbering of *DNMT3A* gene was named according to international recommendations for the description of sequence changes given by the Human Genome Variation Society (www.hgvs.org/mutnomen/). Variant description were checked using Mutalyzer program (http://www.LOVD.nl/mutalyzer) (30). The sequence number of the *DNMT3A* gene corresponding to +1 is the A of the ATG translation initiation codon. The codon number 1 is the initiation codon.

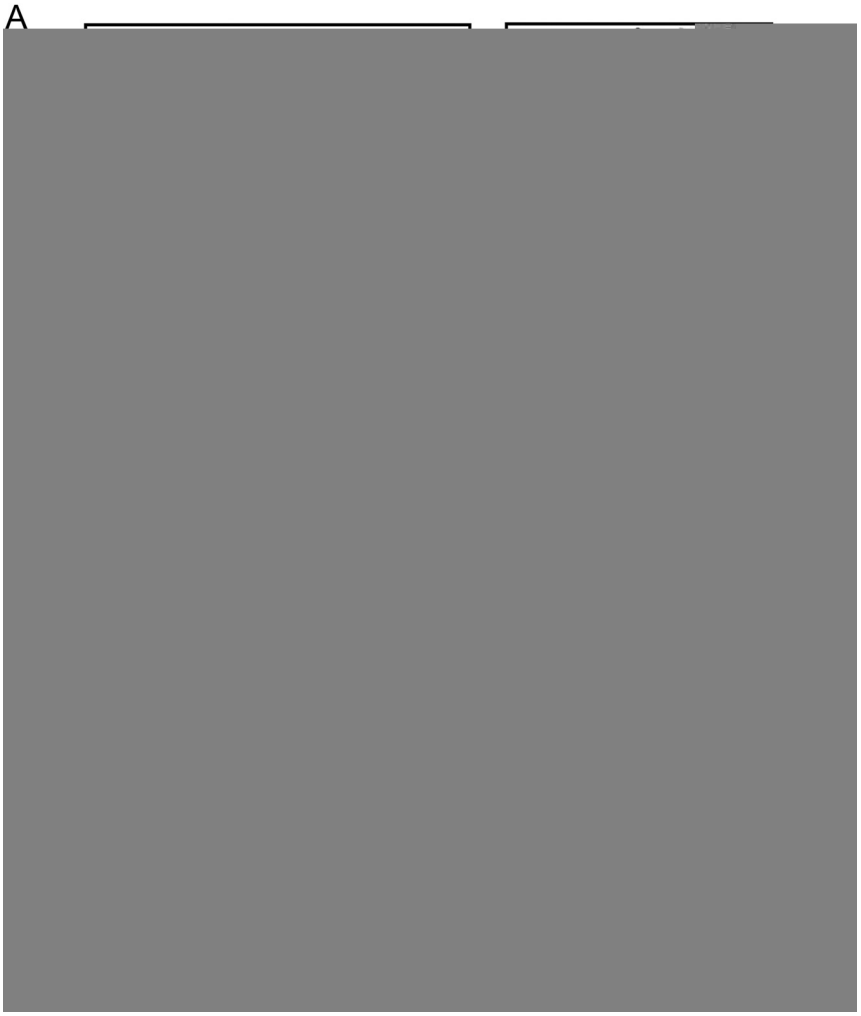

FIG. 2. A, *AR* gene expression (molecular mass 110 kDa) analyzed by Western blotting in foreskin tissue from patients with hypospadias and normal children (mean ± SE, $P < 0.001$). B, *AR* gene methylation in foreskin tissue from patients with hypospadias and normal children (methylation index, mean ± SE, $P < 0.006$).

Western blot analysis

Protein extraction

Protein content was obtained after lysis of the cells using a lysis buffer containing a cocktail of protease inhibitors and subsequent centrifugation at 3000 rpm for 10 min at 4 C. Protein concentration of each sample was determined by BCA protein assay reagent (Pierce, Rockford, IL) using BSA as standard. Fifty micrograms of proteins were used for the Western blot analyses.

Immunoprecipitation was performed by the immunoprecipitation kit (Roche Diagnostics, Mannheim, Germany) according to the manufacturer's instructions. In particular, a preclearing step by protein A/G was performed to reduce the background, and then an appropriate amount (5 μl/sample) of anti-p23 monoclonal antibody (Affinity Bioreagents, Golden, CO) was added to 1 ml of the protein samples and left overnight at 4 C on a rocking platform. Samples were finally washed several times and used for Western blot analysis.

Western blot analysis was performed using the following: 1) a specific antiandrogen receptor polyclonal antibody (Santa Cruz Biotechnology, Santa Cruz, CA) recognizing a protein of 110 kDa; 2) a specific anti-DNMT1 polyclonal antibody (recognizing a protein of 190 kDa) preceded by immunoprecipitation with the anti-p23 monoclonal antibody (Affinity Bioreagents); and 3) specific anti-DNMT3A and -DNMT3B polyclonal antibodies (Epigentek Group Inc., Farmingdale, NY) recognizing a protein of 110 and 100 kDa, respectively. Fifty micrograms of protein were applied to a 4–12% precast NuPAGE MOPS gel (Novex, San Diego, CA) and run in parallel with prestained markers (SeeBlue and MagicMark XP; Invitrogen, Carlsbad, CA) to estimate molecular weight. Proteins were then transferred to nitrocellulose membranes and blocked in 5% nonfat milk for 1 h. Immunoblotting was performed at 4 C overnight, using the specific antibodies. After washing, the blots were incubated at room temperature with peroxidase-conjugated antirabbit or antimouse immunoglobulin antibodies as appropriate (Dako, Carpinteria, CA) at 1:4000 dilutions. Blots were washed and exposed to chemiluminescence solution for 1 min (enhanced chemiluminescence kit; Amersham Life Sciences, Piscataway, NJ), followed by exposure to X-OMAT AR films (Eastman Kodak Co., Rochester, NY). The OD bands were analyzed by the UN-SCAN-IT gel software (Silk Scientific Inc., Orem, UT) and normalized against the amount of proteins loaded.

In vitro studies: fibroblasts primary cultures

Foreskin tissue samples obtained from normal children undergoing circumcision were placed on a 100-mm tissue culture dish and cut them into small pieces using a sur-

gical scalpel. Five to 10 skin pieces were then placed in the center of a 35-mm tissue culture dish, covered with complete growth medium (DMEM, 10% fetal bovine serum, 15 mM HEPES without $NaHCO_3$) and left in a humidified 37 C, 5% CO_2 incubator for 5–6 d. When the cells reached almost confluence, the tissue pieces were eliminated and DMEM medium plus 10% fetal bovine serum with no HEPES was added to the dishes. Once reaching confluence, fibroblasts were trypsinized and divided for further experiments.

In vitro studies: steroid modulation of *AR* gene expression and methylation

Fibroblasts were stimulated *in vitro* with graded doses of DHT and T (10^{-10} to 10^{-6} M). Genomic DNA was isolated from cells incubated with the different testing substances for 72 h and then used for *AR* gene expression and methylation analysis performed by real-time PCR after sodium bisulfite treatment, as described above (20).

In vitro studies: steroid modulation of DNMT

Fibroblasts were stimulated *in vitro* with graded doses of DHT (10^{-11} to 10^{-6} M). Protein content was obtained after lysis of the DHT-stimulated cells for 72 h, using a lysis buffer and subsequent centrifugation, as described above (20).

To assess whether a cochaperone for the heat shock protein 90 chaperoning pathway of steroid receptors, p23, which binds the C terminus of DNMT1 (31, 32), modulates the catalytic activity of DNMT1 and/or contributes to transcriptional repression, p23-AR content in fibroblasts from normal children was evaluated at baseline and after incubation with DHT. A similar amount of proteins was used for immunoprecipitation using an anti-p23 monoclonal antibody (5 μl/sample), following the instructions of the immunoprecipitation kit by Roche Molecular Biochemicals. After electrophoresis, the gel was transferred onto a nitrocellulose membrane and subsequently blotted with an anti-DNMT1 (1:1000) and anti-AR (1:1000) polyclonal antibody, respectively.

At the same time, 50 μg of proteins obtained from the same set of stimulated cells was blotted using a specific anti-DNMT3A (1:400) or -DNMT3B (1:400) polyclonal antibody. The AR expression was also analyzed in the same cells by means of an anti-AR (1:1000) polyclonal antibody.

Statistical analysis

All experiments were repeated on at least three independent occasions. Values are reported as mean ± SE. A test for normality was performed on all data. Statistical significance between the two groups was determined by the *t* test or the Mann-Whitney rank sum test, as appropriate. Comparisons among groups of stimulated fibroblasts were performed using the one-way ANOVA followed by Scheffé multiple comparisons tests or the Kruskal-Wallis one-way ANOVA on ranks. $P < 0.05$ was considered significant.

Results

Hormone serum levels

DHT blood concentrations in patients with hypospadias were significantly lower than in control children (39.48 ± 7.1 *vs.* 137.63 ± 18.7 pg/ml, $P = 0.001$).

CAG microsatellite analysis

The number of CAG repeats ranged from 18 to 30 and from 16 to 27 in blood samples and from 13 to 30 and from 16 to 29 in foreskin tissue samples of patients with hypospadias and normal children, respectively (Fig. 1). Due to the similarity of the CAG ranges, the mean number of CAG repeats both in PBLs (22.6 ± 1.6 *vs.* 21.6 ± 0.9, mean ± SE) and foreskin tissues (21.6 ± 1.1 *vs.* 21.9 ± 1.0) was not different in patients with hypospadias and controls.

AR expression and methylation analysis

The *AR* gene expression in foreskin tissues from patients with hypospadias was lower than in normal children (relative quantification = 0.12). In particular, hypospadic patients showed a 31% reduction in *AR* gene expression in comparison with control children. AR protein content analyzed by Western blotting in foreskin tissue of hypospadic patients was lower than in controls (OD: 32510.0 ± 4719.7 *vs.* 95650.6 ± 4422.8, mean OD ± SE; $P < 0.001$) (Fig. 2A), with a 34% reduction.

The *AR* gene methylation in foreskin tissues from patients with hypospadias was higher than in normal children (methylation index: 69.3 ± 2.7 *vs.* 50.8 ± 4.0, mean ± SE; $P = 0.006$) (Fig. 2B).

In vitro studies aimed at evaluating the potential modulation of *AR* gene methylation by steroids showed that

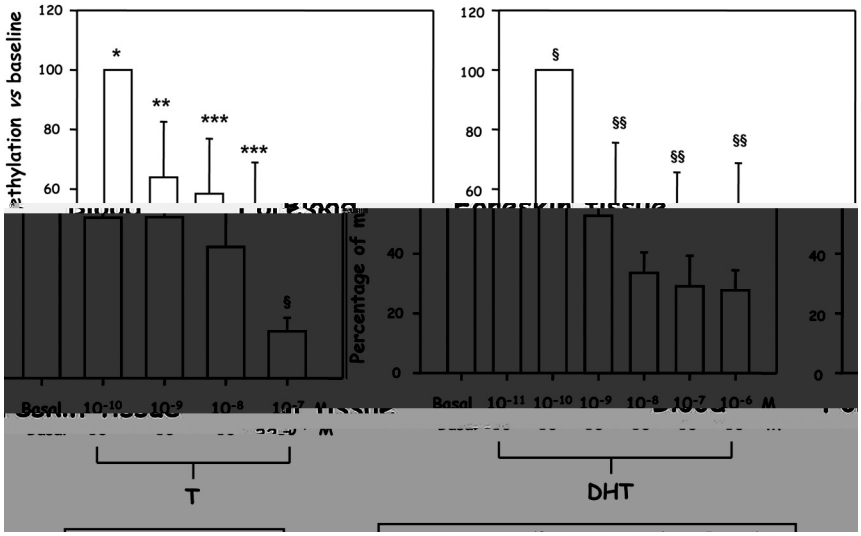

FIG. 3. Modulation of *AR* gene methylation by DHT (one way ANOVA, $P < 0.001$, *, *vs.* all; **, *vs.* 10^{-10} M; ***, *vs.* 10^{-8}, 10^{-7}, 10^{-6} M) and T (one way ANOVA, $P < 0.001$; §, *vs.* all, §§, *vs.* 10^{-7} M).

both DHT and T significantly reduced *AR* gene methylation in a dose-dependent fashion (one way ANOVA, $P < 0.001$) (Fig. 3). The maximal effect was obtained with the greatest T and DHT dose used (10^{-7} and 10^{-6} M, respectively), which yield an 85 and 73% reduction in *AR* gene methylation.

DNMT expression and modulation by steroids

DHT determined a dose-dependent reduction of the p23-AR complex after immunoprecipitation with p23 antibody due to the unfolding of the complex allowing the binding of the ligand to the receptor. No signal was recorded when the p23-DNMT1 complex was analyzed, indicating no functional interaction between p23-AR and DNMT1.

When we analyzed the *DNMT3A* and *DNMT3B* gene expression by Western blotting in *in vitro* fibroblasts, we could not detect any *DNMT3B* gene signal. Conversely, DHT caused a dose-dependent significant reduction of the *DNMT3A* gene expression, with the maximal reduction detected at 10^{-7} M DHT concentration (Fig. 4A). Concomitantly with the dose-dependent reduction of the *DNMT3A* gene expression, DHT also caused an increase in the *AR* gene expression, with the maximal effect detected at 10^{-7} M DHT concentration (Fig. 4B).

In foreskin tissues, the *DNMT3A* gene was expressed more in patients with hypospadias with a 33% increase compared with normal children (OD: 47351.7 ± 3680.4 *vs.* 35507.3 ± 2327.7, mean OD ± SE; $P = 0.013$) (Fig. 5).

Sequencing analysis of the 23 exons of the *DNMT3A* gene and the adjacent splice sites in foreskin and blood samples of hypospadic patients did not reveal any changes in the coding sequence. Among the five described polymorphisms (2010 Ensembl release 59. WTSI/EBI), only the rs2276598 variation was found in both patients and controls.

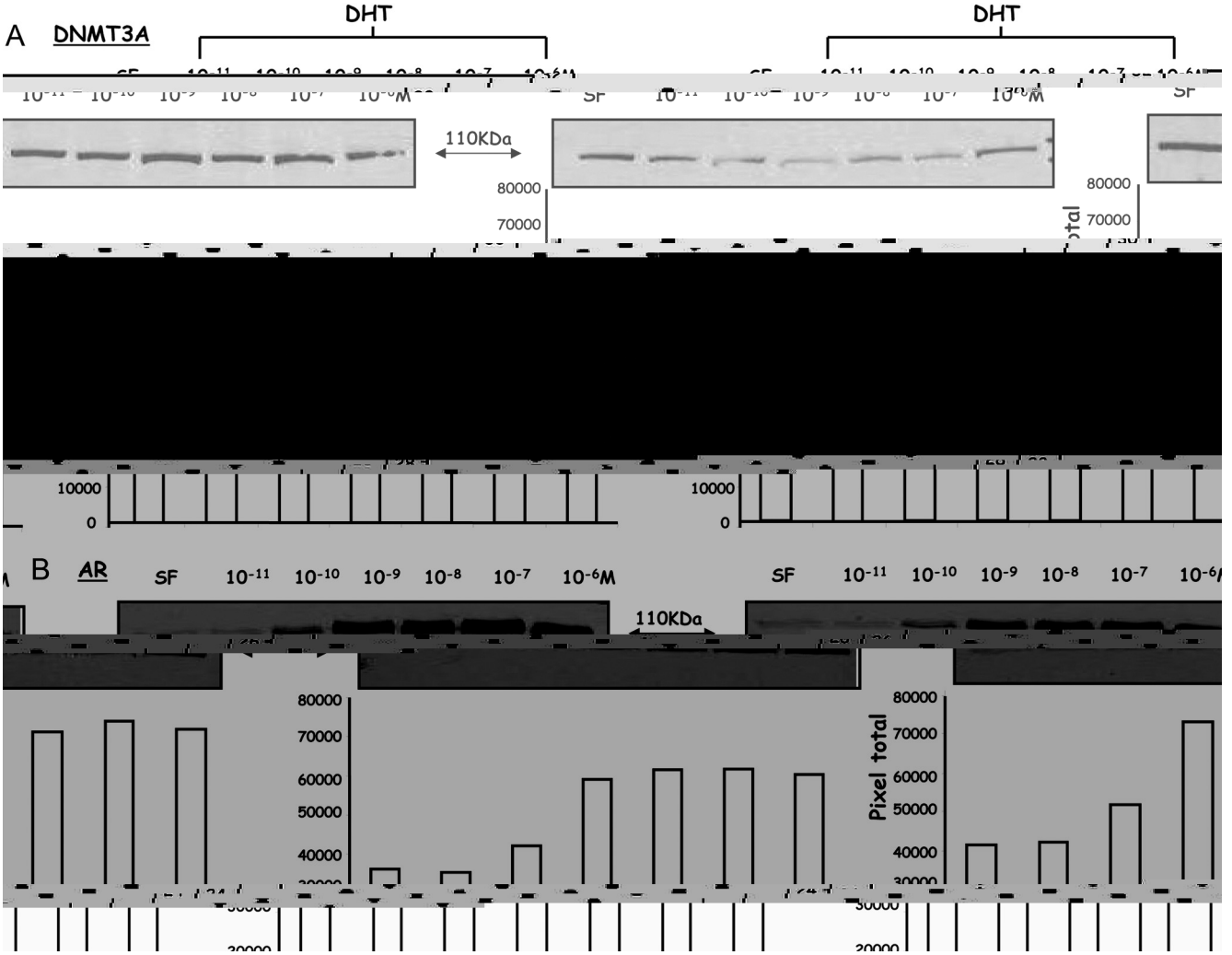

FIG. 4. A, *DNMT3A* gene expression (molecular mass 110 kDa) analyzed by Western blotting in fibroblasts treated with DHT. B, *AR* gene expression (molecular mass 110 kDa) analyzed by Western blotting in fibroblasts treated with DHT.

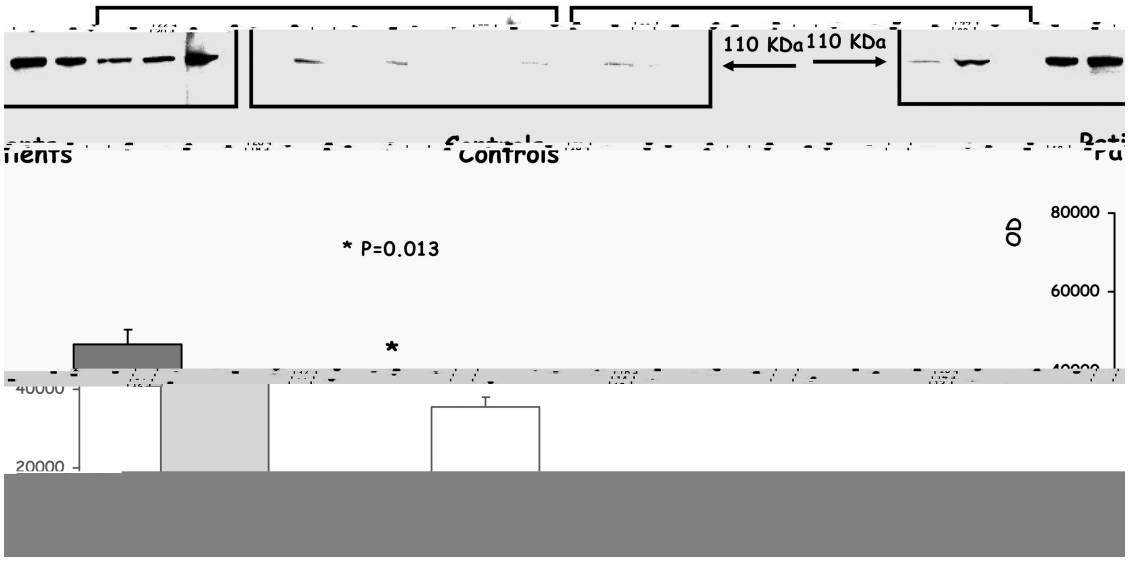

FIG. 5. *DNMT3A* gene expression (molecular mass 110 kDa) analyzed by Western blotting in foreskin tissue from patients with hypospadias and normal children (mean ± SE, $P = 0.013$).

Discussion

The results of the present study indicate that an alteration in the methylation pattern of the *AR* gene, leading to an abnormal *AR* gene expression in target tissue from patients with isolated glandular hypospadias, may contribute to the development of this genital malformation. Specifically, the increased *AR* gene methylation observed in patients with isolated glandular hypospadias resulted in a decreased gene expression that seems to be mediated by DNMT3A. In accordance with previous studies (34–36), no significant differences in the number of CAG repeats, in exon 1 of the *AR* gene, were detected between patients with hypospadias and control children.

Although some limitations of the study due to the number of children analyzed, the selection of isolated glandular hypospadias, and the lack of a complete hormonal study, to our knowledge, this is the first report of a potential involvement of *AR* gene methylation in the pathogenesis of hypospadias. Glandular hypospadias was selected because severe hypospadias (*i.e.* penoscrotal, scrotal, and perineal hypospadias) is often associated with other congenital malformations, and often it is not possible to reach an etiological diagnosis (2).

To clarify the mechanisms underlying the altered *AR* gene methylation pattern in patients with hypospadias, we analyzed the tissue expression of the different genes encoding for the enzymes of the DNMT family (1, 3A, 3B) responsible of the methylation process on the C5 position of cytosines in CpG islands (12). DNMT1 is essential for cell survival and mammalian development, preferentially methylates hemimethylated CpG palindromes in the DNA, and is referred to as a maintenance DNA methylation enzyme. In its C-terminal portion, in which the catalytical center is contained, a binding domain for p23, a cochaperone for the heat shock protein 90 chaperoning pathway of steroid receptors, has been described. Because it is still unclear whether p23 modulates the catalytic activity of DNMT1 and/or contributes to transcriptional repression, we tested the hypothesis that p23 might be involved in the regulation of DNMT1 and in turn in *AR* gene methylation through a possible double link to DNMT1 and AR. No functional interaction between p23 and DNMT1 was detected, suggesting that DNMT1 is not involved in the modulation of the AR methylation.

When the expression of the other members of the *DNMT* gene family was tested, including *DNMT3A* responsible for *de novo* DNA methylation, increased levels of the latter were detected in tissues from hypospadic patients in comparison with controls, indicating that DNMT3 might be involved in generating the increased *AR* gene methylation in hypospadias patients. DNMT3A mutational screening did not show any causative alteration of the coding sequence, indicating that its increased expression might be due to mutational events on promoter site or posttrascritional events that can be influenced by environmental factors, including circulating hormone levels. The results from our *in vitro* studies demonstrate that the stimulation of fibroblasts with increasing doses of androgens causes a dose-dependent reduction of the *DNMT3A* gene expression and a diminished *AR* gene methylation, which is followed by an increased transcriptional activity of the gene itself and therefore an increased level of AR.

We decided to measure only baseline DHT serum concentrations either because we could not get sufficient amount of blood for further hormone measurements or based on the fact that masculinization of the external gen-

italia requires DHT (5). The detection of reduced circulating DHT levels in our patients with hypospadias, not previously described, are in favor of the hypothesis that reduced androgen levels might also be present during the early phase of gestation, which is crucial for sexual differentiation, leading to the increased *AR* gene methylation and thus to the reduced receptor activity. Although results of previous studies did not show evidence of defects in androgen biosynthesis and therefore in the secretion of DHT, in children with hypospadias (37), more recent studies analyzing genes driving to androgen synthesis (38) do support a role of defects in the prenatal androgen biosynthetic pathway in the pathogenesis of hypospadias (39). Defects in 5α-reductase type 2, an enzyme that converts T to DHT, have been identified in severe variants of hypospadias in combination with other genital abnormalities (40, 41).

Other defects in the androgen synthesis pathway are secondary to abnormalities in Leydig cell development or enzymatic defects in testosterone synthesis. These defects are characterized by low concentrations of plasma testosterone in the neonatal period and include mutations of the LH receptor (Leydig cells hypoplasia) (42), a deficit in 3β-hydroxysteroid-dehydrogenase (43, 44), a defect in 17-hydroxysteroid-reductase (45, 46), and rare defects in steroidogenic acute regulatory protein and CYP11A1 (P450 side chain cleavage). The majority of fetal testosterone synthesis and secretion is under the control of placental human chorionic gonadotropin. Thus, a precocious alteration of the placenta or of the maternal-placental unit with insufficient human chorionic gonadotropin secretion might potentially lead to reduced fetal androgen levels and therefore be crucial for the development of hypospadias (47). Such a mechanism has been hypothesized in children born small for gestational age (48–52) as well as in syndromes characterized by hypospadias and placental insufficiency. In our series, the birth weight of children with hypospadias was appropriate for gestational age and not significantly different from that of normal control children. This does not rule out the possibility that a transitory early placental insufficiency, not lasting enough to affect birth weight, might have contributed to the development of hypospadias. If a role of androgens in the *AR* gene hypermethylation of hypospadias can be plausible, the opposite can also be true, *i.e.* that the reduction in androgen biosynthesis is a consequence of the increased *AR* gene methylation.

A pathogenetic role giving rise to reduced androgen levels might be also due to the intrauterine exposure to endocrine disrupters that can exert antiandrogenic activities. Numerous *in vivo* studies have shown that exposure to these toxics is associated to an increased incidence of hypospadias (38, 53). These substances may act in different ways. Some of them, such as phthalates [di (2-ethylhexyl) phthalate and di-*n*-butyl phthalate] (54) and pesticides (procloraz) (33) can inhibit the fetal synthesis of testosterone, causing an androgenic depletion in the fetus, and an increased AR gene methylation, combining the two mechanisms. It is in fact, very likely the correlation between the increased number of hypospadias in the last 25–30 yr in well-developed countries and the increased level of environmental pollution present in these areas (53). However, further studies are necessary to define which are the levels of toxicity of the endocrine disrupters, whether they are able to determine the development of fetal and neonatal genital malformations, and which are the mechanisms responsible for these alterations.

In conclusion, foreskin tissues of patients with hypospadias show an increased *AR* gene methylation mediated in part by the enzyme DNMT3A, which has a role in *de novo* methylation. The *AR* gene hypermethylation might lead to a decrease in *AR* gene expression, and this could play a role in the development of genital malformation. Further studies are necessary to assess whether this epigenetic abnormality represents the cause of hypospadias or is secondary to an altered fetal steroid milieu. In addition, it would be interested to extend this analysis to patients with severe hypospadias, including the study of other genes associated with hypospadias.

Acknowledgments

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Disclosure Summary: The authors have nothing to disclose.

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