Androgen Receptor Mutations Are Associated with Altered Epigenomic Programming as Evidenced by HOXA5 Methylation

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**Key Words**
Androgen insensitivity syndrome · Androgen receptor mutation · DNA methylation · Epigenetic · HOXA5

**Abstract**

Male external genital differentiation is accompanied by implementation of a long-term, male-specific gene expression pattern indicating androgen programming in cultured genital fibroblasts. We hypothesized the existence of an epigenetic background contributing to this phenomenon. DNA methylation levels in 2 normal scrotal fibroblast strains from 46,XY males compared to 2 labia majora fibroblast strains from 46,XY females with complete androgen insensitivity syndrome (AIS) due to androgen receptor (AR) mutations were analyzed by Illumina GoldenGate methylation arrays\(^\circ\). Results were validated with pyrosequencing in labia majora fibroblast strains from fifteen 46,XY patients and compared to nine normal male scrotal fibroblast strains. HOXA5 showed a significantly higher methylation level in complete AIS. This finding was confirmed by bisulfite pyrosequencing of 14 CpG positions within the HOXA5 promoter in the same strains. Extension of the 2 groups revealed a constant low HOXA5 methylation pattern in the controls in contrast to a highly variable methylation pattern in the AIS patients. HOXA5 represents a candidate gene of androgen-mediated promoter methylation. The constantly low HOXA5 DNA methylation level of normal male scrotal fibroblast strains and the frequently high methylation levels in labia majora fibroblast strains in AIS indicate for the first time that androgen programming in sexual differentiation is not restricted to global gene transcription but also occurs at the epigenetic level.

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Androgens are a special type of developmental hormone because they usually exert their biological effects via 2 timelines. On the one hand, androgens activate reversibly androgen receptor-mediated signaling in target cells [Werner et al., 2006]. On the other hand, there are certain sensitive windows during early embryogenesis and later post-natal life where androgens typically lead to irreversible anatomic changes thus resulting in the implementation of the normal male genital phenotype. Additional biological examples for irreversible androgen actions during male development are the pubertal changes of body shape including the male face [Schaefer et al., 2005] and growth of the larynx resulting in a deepening of the voice [Pedersen et al., 1986; Harries et al., 1998]. There is evidence for a sex-specific brain development and a particular role of prenatal programming of androgens therein [Döhler, 1985–1986; Hines, 2003; Jürgensen et al., 2007; Swaab, 2007].

More than 400 different AR mutations associated with AIS have been identified to date (http://www.mcgill.ca/androgendb/); however, there is not always a clear correlation between AR mutation and clinical phenotype, mainly in PAIS [Evans et al., 1997; Boehmer et al., 2001b]. As possible causes of the phenotypic heterogeneity, AR somatic mosaicism, AR cofactors and differences in 5α-reductase 2 activity were proposed in some cases [Holterhus et al., 1997, 1999; Adachi et al., 2000; Boehmer et al., 2001a]. A genome-wide approach unraveled the existence of sex-dimorphic gene expression patterns in cultured scrotal fibroblast strains of normal 46,XY individuals compared with 46,XY AIS patients [Holterhus et al., 2003, 2007]. While the gene expression patterns reflected differences in androgenization of the genitalia, the functional link between AR mutation and altered gene expression patterns remained unclear.

In normal AR signaling, dihydrotestosterone is bound to the ligand binding domain (LBD) of the AR. This leads to a conformational change of the LBD which in turn leads to the release of molecular chaperones and to the recruitment of distinct coregulatory factors including enzymes that covalently modify histones and remodel chromatin [McEwan, 2004; Jia et al., 2006]. Comprehensive studies of the AR target gene KLK3 (kallikrein-related peptidase 3) revealed significant increase in histone H3-K9/K14 acetylation and H3-K4 methylation in androgen-independent prostate cancer cells compared to their androgen-dependent counterparts [Jia et al., 2006]. Epigenetic changes at the DNA level therefore contribute to the control of transcription by androgens and the AR. This concept of hormonal control was also proven by a recent study that identified DNA methylation and demethylation by hormonal switching as main regulator for the function of the CYP27B1 gene promoter [Kim et al., 2009].

In an approach to identify epigenetic mechanisms that translate androgen receptor mutation into androgen-dependent gene expression patterns, we here compared the DNA methylation of labia majora fibroblast strains from 46,XY patients with AIS to scrotal fibroblast strains from normal 46,XY males.

**Materials and Methods**

The study has been approved by the ethical committee of the Christian Albrechts University of Kiel.

**Fibroblast Strains, Cell Culture and Treatment Conditions**

Scrotal-derived fibroblast strains were obtained from nine 46,XY males. Labia majora- or labioscrotal-derived fibroblast strains were obtained from fifteen 46,XY patients with AIS and proven inactivating mutations of the AR gene and two 46,XY patients with AIS lacking a mutation in the AR gene as previously described [Holterhus et al., 2003, 2007]. Fibroblasts were cultured to confluency on 150-mm plastic dishes at 37°C with 5% CO₂. They were maintained in phenol-red-free DMEM F12 (Dulbecco’s Modified Eagle Medium with the nutrient mix F12, Gibco/Invitrogen, Karlsruhe, Germany) containing l-glutamine, 15 mM Hepes buffer, penicillin/streptomycin (Gibco) and 12.9% fetal calf serum (FCS, Gibco). Passage number of fibroblast culture and age at biopsy did not significantly differ in the 2 patient groups characterized by HOXA5 DNA promoter hypermethylation and HOXA5 DNA promoter hypomethylation.

**DNA Isolation**

DNA was isolated from fibroblasts using the DNeasy Blood & Tissue protocol (Qiagen, Hilden, Germany).

**DNA Methylation Profiling Using Universal Bead Arrays**

Using the GoldenGate Methylation Cancer Panel I® (Illumina Inc., San Diego, Calif., USA) we have performed a pilot study to identify CpG loci differentially methylated between 2 AIS patients and 2 healthy controls. Both patients had CAIS due to inactivating AR mutations. DNA methylation analyses were done as described previously [Martin-Subero et al., 2009a, b]. The array allows assaysing 1,505 CpG sites from 807 selected genes, primarily focusing on genes relevant for tumorigenesis, differentiation, cell cycle control, and apoptosis. The reproducibility and accuracy of the GoldenGate Methylation Cancer Panel I based DNA methylation analysis has been demonstrated extensively [Bibikova et al., 2006; Martin-Subero et al., 2009b]. All analyses were

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*HOXA5 Methylation in AIS*
performed in duplicate. CpG loci showing a delta beta value of at least 0.3 and an Illumina DiffScore above 30 or below –30 were considered as differentially methylated. The complete dataset is provided in online supplement table 1 (for all online suppl. material, see www.karger.com/doi/10.1159/000323807).

**Bisulfite Pyrosequencing**

Bisulfite pyrosequencing analysis of HOXA5 promoter methylation was performed according to standard protocols with slight modifications [Tost and Gut, 2007]. Genomic DNA was bisulfite converted using the EpiTect Bisulfite Conversion Kit (Qiagen). In a subsequent PCR amplification, locus-specific primers were used with one primer biotinylated at the 5’ end (5’-TTT TTG GTA AGT TTG GAA GTT AGG ATT TT-3’, 5’-AAA ACC CCA ACA AAA CCC AAC AT CT-3’). Amplification reactions contained approximately 75 ng bisulfite-converted DNA, primers, AccuPrime Taq Polymerase, buffer II (Invitrogen), 50 mM MgCl₂, and 2.5 mM of each dNTP in a final volume of 50 µl. After denaturation, PCR consisted of 45 cycles of each 95°C for 30 s, 60°C for 30 s, and 68°C for 30 s, followed by a final synthesis at 68°C for 2 min. PCR products were verified by gel electrophoresis. Single strands were prepared using the Vacuum Prep Tool (Biotage, Uppsala, Sweden) followed by a denaturation step at 85°C for 2 min and final sequencing primer (5’-GGT TTT GAA AGT TGT G-3’) hybridisation. Pyrosequencing was performed using the Pyrosequencer ID and the DNA methylation analysis software Pyro Q-CpG 1.0.9 (Biotage), which was also used to quantify the ratio T:C (mC:C) at the analyzed CpG sites. Assays were validated using a commercial in vitro methylated DNA as positive control and whole genome amplification DNA as negative control (GE Healthcare, Munich, Germany). Principle component analysis has been performed using the Omics Explorer, Version 2.0 Beta (Quacore AB, Lund, Sweden).

**RNA Preparation**

Total RNA was isolated from fibroblasts using Trizol (Invitrogen, Paisley, UK) followed by RNeasy mini kit protocol (Qiagen) according to the manufacturers’ protocols. DNA contamination was removed using the DNA-free kit (Ambion, Austin, Tex., USA). Total RNA was quantified using the 2100 Bioanalyzer (Agilent, Palo Alto, Calif., USA).

**qRT-PCR**

Total RNA was amplified according to the manufacturer’s instructions using the First Strand cDNA Synthesis kit (Fermentas, Hannover, Germany). qRT-PCR was performed using the IQ™ SYBR Green Supermix PCR kit (Bio-Rad Laboratories, Munich, Germany). The primers used for PCR (biomers.net GmbH, Ulm, Germany) had the following sequences: HOXA5 forward primer 5’-GCA CAT AAG TCA TGA CAA CAT AG-3’, and HOXA5 reverse primer 5’-CAG TAC TTT TTT AAA CGC TCA GAT AC-3’. Hydroxymethylbilane synthase (HMBS) was used as housekeeping gene for normalization. HMBS forward primer was 5’-CTT CAC CAT CGG AGC CAT CTG C-3’ and HMBS reverse primer was 5’-CGA AGC CGG GTG TTG AGG TTT TTT-3’. PCR was carried out with the iCycler real time PCR detection system (Bio-Rad). We performed 2 independent experiments, each carried out as duplicates resulting in 4 HOXA5 Ct values and 4 HMBS Ct values for each individual. For statistic analysis the mean Ct value for HOXA5 and HMBS was used for each individual.

**Results**

**Array-Based Methylation Analysis**

Array-based DNA methylation analysis of 1,505 CpGs representing 807 genes using the Illumina GoldenGate Methylation Cancer Panel I demonstrated low DNA methylation values of HOXA5 at positions chr7: 27,149,625 bp and chr7: 27,151,136 bp (NCBI Build 36.1). Avg-beta: methylation value. Patient: labia majora fibroblast strains from two 46,XY patients with CAIS due to AR mutations. Control: 2 scrotal fibroblast strains from 46,XY male controls. All analyses were performed in duplicate.

For a 50-µl PCR master mix 25 µl of IQ™ SYBR Green Supermix, 2 µl of forward and reverse primer, respectively, 1 µl of cDNA and water were mixed. After an activation step of 95°C for 7 min, 40 amplification and detection cycles were performed (95°C for 30 s, 60°C for 45 s and 70°C for 1 min). Melting curves were analyzed with the iCycler iQ optical System Software Version 3.0a (Bio-Rad). Fold changes were calculated using the ∆Ct method [Bookout et al., 2006].

**Bisulfite Pyrosequencing of the HOXA5 Promoter Region**

Quantitative methylation analysis of 13 CpG positions in the promoter region of HOXA5 surrounding the CpG
at position chr7: 27,151,136 bp which was previously investigated on the methylation array with bisulfite pyrosequencing revealed a mean DNA methylation range from 16 to 33% in 9 male 46,XY scrotal fibroblast strains (overall mean methylation 28%). There was little variation reflected by a standard deviation of 5%. The methylation analysis of 5 labia majora (CAIS), 9 labioscrotal (PAIS) and 1 scrotal (MAIS) fibroblast strains from 46,XY patients with AR mutations and various degrees of AIS (according to table 1 in Holterhus et al. [2007]) revealed a striking variability of methylation patterns ranging from 14 up to 82% mean DNA methylation per case (overall mean methylation 41%) with a standard deviation of 20% (online suppl. fig. 1). Thus, constantly low HOXA5 DNA methylation levels of normal male 46,XY scrotal fibroblast strains are switched to high variability of HOXA5 DNA methylation levels in labioscrotal fibroblast strains of patients with AR mutations and various degrees of AIS phenotype.

Unsupervised hierarchical cluster analysis of the HOXA5 DNA methylation values derived from bisulfite pyrosequencing analyses identified 2 different groups within all tested individuals. Remarkably, all 9 male controls and 8 of 15 AIS patients (2 CAIS, 2 AIS4, 1 AIS3, and 3 AIS2 patients) showed low HOXA5 DNA promoter hypermethylation. In contrast, 7 from 15 AIS patients (3 CAIS, 1 AIS3, 2 AIS2 and 1 MAIS patient [Scrotum 8; Appari et al., 2009]) but none of the normal male controls showed HOXA5 DNA hypermethylation (fig. 2). The patients with AR mutations significantly more frequently showed high

**Fig. 2.** Top: Unsupervised hierarchical cluster analysis of the methylation studies from 14 CpG positions within the HOXA5 promoter region of labia majora fibroblast strains from fifteen 46,XY patients with AIS and documented AR mutation and scrotal fibroblast strains from nine 46,XY male controls. Included is the information of AR/AR mutation and genital phenotype for each individual tested. AR mutations are signed according to the androgen receptor gene mutation database world-wide web server (http://androgendb.mcgill.ca/). CAIS: complete androgen insensitivity syndrome, female external genitalia; AIS4: slight virilization, predominantly female external genitalia; AIS3: ambiguous external genitalia; AIS2: predominantly male external genitalia; MAIS: male external genitalia. Red: high methylation level; green: low methylation level. Red squares indicate patients with various AIS degrees and documented AR mutations. The green square indicates Scrotum 8, an individual with an AR mutation (c.2884T>G, p.I841S) but normal male external genitalia (MAIS). Blue squares indicate normal male controls. Bottom: Results of a principle component analysis (PCA). PCA has been performed using the Omics Exporer, Version 2.0 Beta. PCA significantly separates a patient subgroup with HOXA5 DNA promoter hypermethylation from all male controls and a second subgroup of patients.
methylation values than normal controls (p = 0.02, Fisher's exact test).

Out of the group with low HOXA5 DNA methylation, there is 1 CAIS patient, who is known to be mosaic for an AR mutation (ARD 465, p.E287X [Holterhus et al., 2003]).

One individual with normal male phenotype, who initially presented with infertility, carried an androgen receptor mutation (c.2884T>G, p.I841S [Appari et al., 2009]). Remarkably, despite the normal male genital phenotype he showed an extremely high HOXA5 promoter methylation pattern with a mean methylation value of 82%. The identical AR mutation (c.2884T>G, p.I841S) was also observed in an AIS4 patient who showed a mean HOXA5 promoter methylation of only 20%. These results indicate that there is no direct correlation between a given AR mutation and HOXA5 DNA methylation levels in the analyzed fibroblast strains of the corresponding individuals.

Moreover, 2 additional patients with AIS (CAIS, AIS3) who lacked an androgen receptor mutation by sequencing [Holterhus et al., 2005] showed very low HOXA5 promoter methylation patterns with mean methylation values of 11 and 14%, similar to the HOXA5 DNA methylation levels of the 9 male controls (data not shown). Thus, no obvious association of the HOXA5 DNA methylation level in the analyzed fibroblast strains and the AIS phenotype was detectable.

qRT-PCR of HOXA5

We studied expression of HOXA5 transcripts in 8 male control scrotal fibroblast strains and 14 AIS labioscrotal fibroblast strains by qRT-PCR. HOXA5 methylation of the CpG loci under study did not lead to HOXA5 gene repression. Instead, we observed a significant (p = 0.01) linear relationship between increasing HOXA5 DNA methylation values and increasing HOXA5 expression with an r of 0.52 and an r² of 0.271.

Discussion

Here we describe HOXA5 as significantly hypermethylated in 2 CAIS patients compared to 2 normal male controls in an explorative screening of global genital fibroblast methylation status using the GoldenGate Methylation Cancer Panel I microarray. Given the involvement of HOXA5 as member of a conserved family of transcription factors in embryogenesis, tissue-specific development and cell differentiation [Scott et al., 2005], its interplay with hormones and its influence in development of reproductive tissues through its downstream target genes IGFBP1, PTN and the progesterone receptor PGR [Svin- gen and Tonissen, 2006], HOXA5 seemed to be a promising candidate gene for further investigation of DNA methylation in AIS. Therefore, we performed an independent validation of our methylation array experiments in fifteen 46,XY labia majora and labioscrotal fibroblast strains from patients with documented AR mutations and various degrees of AIS, as well as in 9 scrotal fibroblast strains from 46,XY male controls. Hershko et al. [2002] previously reported that methylation of HOXA5 occurred only in CpG island sequences. Thus, our bisulfite pyrosequencing study design was set up accordingly by analyzing 14 CpG positions in the CpG island of the HOXA5 promoter region.

We could reproduce the results for the 4 individuals on the methylation array by bisulfite pyrosequencing, confirming a good correlation between the 2 independent methods used. Moreover, we observed that HOXA5 promoter methylation patterns of all additionally tested male controls remained stably low, while the HOXA5 promoter methylation patterns of the AIS patients revealed a striking variability with 7 of 15 AIS patients showing HOXA5 hypermethylation and 8 of 15 patients showing low HOXA5 methylation levels. These observations could support the hypothesis that HOXA5 promoter methylation is controlled by the androgen receptor and that a constantly low HOXA5 methylation pattern is usually associated with, but not sufficient for, normal male genital development, as can be observed in some CAIS patients having as low HOXA5 methylation levels as normal male controls (fig. 2). On the one hand, this indicates that the AR must have additional influences on other genes beside HOXA5 and is thereby probably contributing on multiple redundant sites in a whole gene network to the virilization deficit in AIS patients. On the other hand, the observed differences of HOXA5 DNA methylation levels even in the case of identical AR mutations (e.g. c.2884T>G, p.I841S) indicate that HOXA5 DNA methylation control is not restricted to the AR gene but rather involves different other genes, probably at different time windows and to a different extent. Thus, our experimental data suggest that androgen-regulated genital differentiation is embedded in a coordinated developmental network involving the androgen receptor as an important, but not exclusive, factor. The phenomenon that transcriptional control of a gene is not restricted to the influence of a single gene is well known due to the fact that e.g. AR-binding sequences (ARE, ARR or HRE) occur next to binding sites for housekeeping (Sp1 [Chen

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et al., 1997], CCAAT and NF-1 [Song et al., 1999]), inducible (NF-κB [Supakar et al., 1995], Ets and AP-1 [McEwan, 2004]) and tissue-specific transcription factors [McEwan, 2004]. However, how the implementations of epigenetic changes occur in the case of HOXA5 and which additional genes beside AR are involved may be entirely unrelated to our current knowledge of androgen-related transcriptional control. Estrogens do not play a leading role in sex-specific morphogenesis of the external genitalia. However, AIS is generally characterized by an increased conversion of testosterone to estradiol by aromatization. Therefore, we cannot exclude that part of the HOXA5 promoter methylation patterns may have been modulated via the estrogen receptor pathway in the AIS samples, thus contributing to the observed epigenetic variability. In the same way, prenatal influences through medication or pregnancy course may affect methylation of the HOXA5 promoter and are of high interest for further studies in the field of AIS phenotypic heterogeneity.

In conclusion, we show that HOXA5 represents a candidate gene of androgen-mediated promoter methylation. We thereby suggest the concept that epigenetic changes may function as long-lasting androgen memory, supporting our earlier genome-wide gene expression studies in fibroblasts and in blood [Holterhus et al., 2003, 2007, 2009]. While we were neither able to link the HOXA5 DNA methylation levels to a distinct AIS phenotype nor to a distinct AR mutation, the coordinated interplay of genes involved in the complex network that contributes to male genital differentiation could possibly explain PAIS heterogeneity (online suppl. fig. 2).

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