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THE FIRST DETECTION OF COMPLETE ANDROGEN INSENSITIVITY WITH NO MUTATION IN THE CODING SEQUENCE OF THE ANDROGEN RECEPTOR GENE

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ABSTRACT

We have analyzed the entire nucleotide sequences of complementary DNAs of the androgen receptor gene in two siblings (patients 8044 and 8047) with complete androgen insensitivity. Plasma testosterone was in the normal male range, however, androgen binding capacity was undetectable as measured in skin fibroblasts in both patients. 5α-Reductase activity was normal in both cases confirming that this enzyme is not involved in the mechanism of androgen insensitivity. Northern blot analysis indicated that mRNA of the AR was normal in size. In addition, no mutation was found in the entire nucleotide sequences of complementary DNAs of the androgen receptor gene. Together, our results reveal an unusual insight into the molecular basis of androgen resistance, and the molecular heterogeneity in this clinical spectrum.

INTRODUCTION

Androgens play an important role in control of the development of the normal male phenotype and the regulation of virilization in the adult. Various actions of androgens are mediated by an intracellular protein, the androgen receptor (AR), which influences the transcription of androgen-responsive genes. AR, a member of the steroid/thyroid receptor superfamily, acts as a *trans*-regulator of transcription upon binding with its cognate ligand. This hormone-receptor complex interacts with *cis*-acting DNA elements to regulate the transcription of its target genes (1). Basically, the structural organization of the AR comprises a variable N-terminal region involved in the modulation of gene expression, a well conserved DNA-binding domain with two zinc fingers, and a partially conserved C-terminal ligand-binding domain (2).

It is noted that absence of a functional AR causes androgen insensitivity syndrome (AIS) with female external phenotypes in 46,XY subjects (3-5). Analysis of the molecular defects of the AR gene in such patients has provided valuable insight into the structural and functional organization of the AR molecule.

In patients with AIS, many mutations in both the DNA- and ligand-binding domains of the AR gene, and just a few cases in the N-terminal region, have been described (3-5). These include mutations in the arginine-614 of the DNA-binding domain and in the valine-888 of the ligand-binding domain in the AR gene of patients with complete androgen insensitivity (CAI) (6, 7). Together, results of these studies have reinforced the concept that AIS is caused by qualitative mutations in the AR gene. Herein, we report, for the first time, the study of two affected siblings among 15 patients with CAI, showing normal wild-type coding sequence of the AR gene with the absence of specific androgen binding.

MATERIALS AND METHODS

Cell culture.

Fibroblast strains were obtained from skin biopsies of patients with AIS and propagated in a 4/1 mixture of medium 199/Dulbecco's modified Eagle's medium (DMEM) supplemented with antibiotics and 10% fetal calf serum as previously described (8).

Androgen-binding capacity.

Androgen-binding capacity was measured as previously described (9) on intact monolayers grown to confluency in 100 mm petri dishes. Replicate culture received increasing concentrations (0.05-2.0 nM) of [^{3}H]-5α-dihydrotestosterone (DHT) in a serum-free medium, alone or with two-hundred-fold excess of non-radioactive DHT. Cells were incubated for 60 min at 37°C under normal culture conditions. At the end of incubation, cells were washed, harvested and nuclear proteins were extracted by sonication in 50 mM Tris-HCl, pH 7.4 buffer containing 1 mM EDTA and 0.6 M KCl. Free and loosely bound DHT was removed by dextran-coated charcoal treatment. Specific binding was calculated as the difference between total binding ([^{3}H]-DHT alone) and nonspecific binding (nondisplaced in the presence of an excess of unlabeled DHT). The maximum binding capacity (B_{max}) and the apparent dissociation constant (K_D) of

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the androgen receptor were derived from Scatchard plot analysis. Binding capacity was expressed as femtomoles of DHT bound per mg DNA. For each patient, binding capacity was measured at least twice in separate experiments. In some experiments, incubations were continued for 18 h to assess up-regulation of the AR. These conditions usually result in a 2- to 3-fold increase of the normal AR (10). DNA was assayed after perchloric acid extraction as previously described (11).

5α-Reductase enzyme assay.

5α-Reductase activity was measured as previously described (9). Cells were cultured in 60 mm Petri dishes containing serum-free medium 24 h before assay. The medium was replaced by 2 ml of serum-free medium containing 5 nM [^{3}H]-testosterone (50 to 60 Ci/mmol) and incubated for 15 min to 2 h (at least 3 time points) at 37°C in 5% CO_2. The dishes were then put on ice to stop the reaction. Fifty µl of medium from each dish was added into counting vials to measure the total radioactivity. To monitor recovery, the medium from individual dish was transferred into a tube containing [^{14}C]-tracers (10,000 cpm of testosterone, 1,000 cpm of DHT, 1,000 cpm of 3α- or 3β-Diol, and 1,000 cpm of androstenedione). The medium from each dish was transferred into another tube containing unlabeled tracers (4 mg/ml ethanol), and extracted by vortexing 1 min with 10 ml of ethyl acetate/cyclohexane 1/1 (v/v). The organic phase was evaporated to dryness and analyzed by thin-layer chromatography in chloroform/methanol 97.5/2.5 (v/v) for 90 min. Testosterone and androstenedione were visualized under UV light (240 nm) and 5α-reduced steroids in iodine vapors. Steroids were scraped from the silica gel plates (Merck, GF254), and eluted in acetone and ethanol. 3α- and 3β-Diols, not separated in this system, were eluted together. Eluates were evaporated to dryness and counted for radioactivity. 5α-Reductase activity was calculated as the amount of DHT and Diols formed in 1 h and expressed as fmol/µg DNA/h.

RNA extraction.

Cells (6 x 10^6) grown to confluency and placed in serum-free medium 24 h before extraction were harvested using trypsin-EDTA, collected in serum-free medium, and centrifuged at 200 x *g* for 15 min. The cell pellet was washed 3 times with sterile saline and transferred into two eppendorf tubes (1.5 ml). The cell pellet was either extracted immediately or stored at -80° C. Extraction was performed using the one-step RNAzol extraction procedure (12). The cell pellet was carefully suspended in 600µl of RNAzol (Bioprobe Systems, France) and 60µl chloroform/isoamylic alcohol (24/1) was added. Tubes were vigorously agitated, allowed to stand 15 min on ice, and centrifuged at 11,750 x *g*, 2° C for 15 min. The aqueous phase was transferred into a clean eppendorf tube and RNA was precipitated in isopropanol for 2 h at -80° C.

Complementary DNA preparation.

The following primers were used for separate analysis of the N-terminal domain (NTD), DNA-binding domain (DBD), and ligand-binding domain (LBD), which cover the entire AR cDNA (6, 7).

primer 5 of NTD: 5'-GTGCAGTTAGGGCTGGGAAGG-3'
primer G of NTD: 5'-CGGGTTCTCCAGCTTGATGCG-3'
primer 2 of DBD: 5'-TCGCGACTACTACAACTTTC-3'
primer 4 of DBD: 5'-TGGCTCAATGGCTTCCAGGA-3'
primer A of LBD: 5'- GTGGAAATAGATGGGCTTGA-3'
primer B of LBD: 5'-TCACACATTGAAGGCTATGA-3'

Complementary DNAs were synthesized using 1µg of total cellular RNA by the GeneAmp RNA PCR Kit (Perkin Elmer, Branchburg, NJ) according to the manufacturer's specifications. Amplification by polymerase chain reaction (PCR) was performed using two-thirds of the cDNA synthesis reaction mixture and the above pairs of primers (100 ng each) in a total volume of 100µl by Perkin Elmer PCR apparatus during 30 cycles. Each cycle included denaturation for 1 min at 95° C, annealing for 2 min at 60° C, and extension for 3 min at 72° C. After the last cycle, samples were incubated at 72° C for 7 min. The amplified products were recovered after phenol/chloroform extraction and ethanol precipitation.

DNA cloning and sequencing.

Amplified cDNAs were cloned by either the TA (InVitrogen, San Diego, CA) or pT7Blue cloning system (Novagen, Madison, WI) as described in the manufacturer's instruction. Sequence was analyzed by the dideoxynucleotide chain termination method (13) in SequaGel system (National Diagnostics, Manville, NJ) directly on plasmid DNA (14).

RESULTS AND DISCUSSION

Patients 8044 and 8047 are affected siblings aged 17 and 18, respectively. They were first seen at the endocrine clinic for primary amenorrhea. Both presented the typically clinical phenotype of CAI with normal female genitalia, harmonious breast development, and the absence of body hair. Examination revealed a short and blunt vagina and the absence of uterus. Both karyotypes were 46,XY and hormonal profiles were summarized in Table 1. Plasma testosterone was 5.5 and 8.2 ng/ml in patients 8044 and 8047, respectively (normal adult male level, 5.8 ± 2.4 ng/ml). Testosterone/DHT ratio was in the normal range for adult males. Plasma delta 4-androstenedione level was 1.1 and 1.2 ng/ml (normal

Table 1. Androgen binding, 5α-reductase and other metabolic activities on normal male and CAI patients

	Normal male	**CAI patients**	
		8044	8047
B_{max} (fmol/mg DNA)	872 ± 283	Not detectable	Not detectable
5α-Reductase (fmol/μg DNA/h)	> 2.1	68 ± 6	2.5
Plasma testosterone (ng/ml)	5.8 ± 2.4	5.5	8.2
Plasma delta 4-androstenedione (ng/ml)	0.90 - 1.20	1.1	1.2
Plasma estradiol (pg/ml)	25 ± 8	30	53
LH (mU/ml)	1-5	15	20
FSH (mU/ml	2-9	12	18
Urinary androstanediol glucuronide excretion (μg/24h)	193 ± 77	74	50

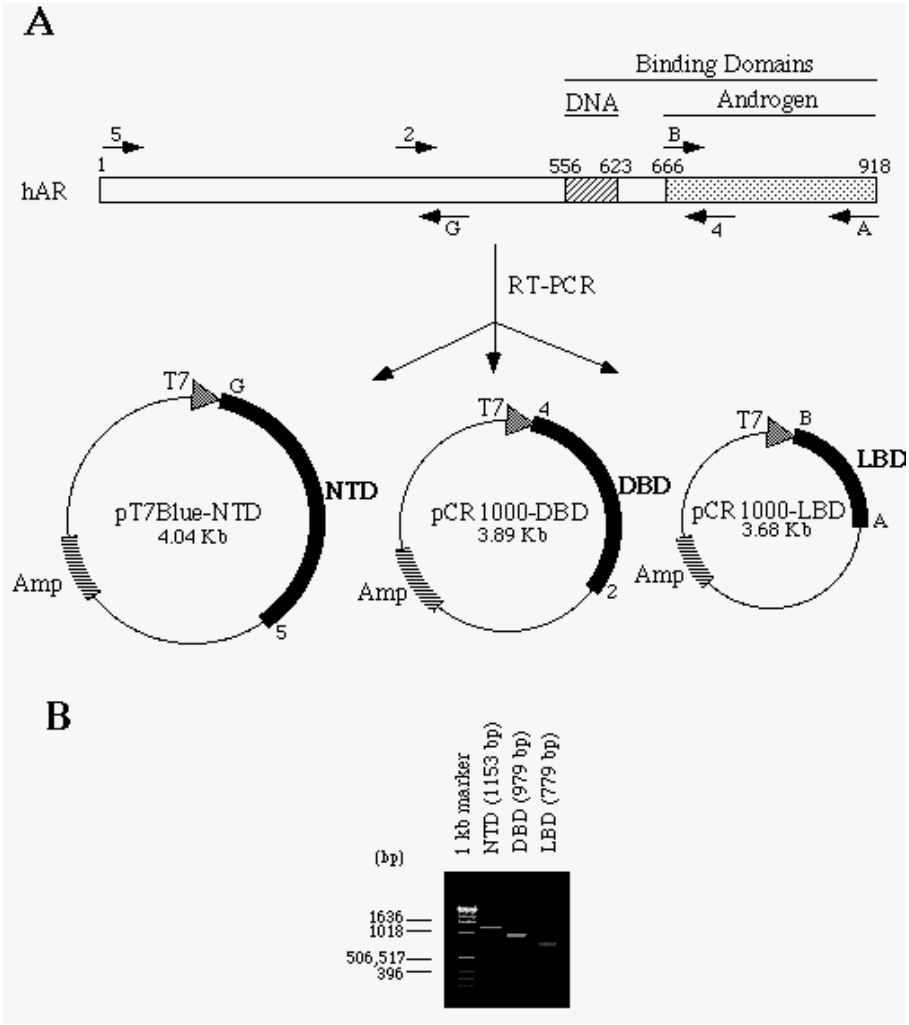

Fig. 1. (A) Schematic structure of the human AR cDNA and plasmids containing each domain amplified from RT-PCR. The entire human AR (hAR) cDNA can be basically divided into three parts, the N-terminal (NTD), DNA-(DBD) and ligand-binding (LBD) domains, flanked by three pairs of primers, 5 and G, 2 and 4, as well as A and B, respectively. (B) RT-PCR analysis. Each domain of the AR was amplified by RT-PCR and directly subcloned into either pCR1000 or pT7Blue vector. Sequence was analyzed by the dideoxynucleotide chain termination method.

adult male range, 0.90-1.20 ng/ml). Plasma estradiol was 30 and 53 pg/ml, which is in the high range value for adult males that may due to the estrogen replacement therapy. LH and FSH levels were high due to negative feedback from lacking of normal AR, 15 and 20 mU/ml for patient 8044, and 12 and 18 mU/ml for patient 8047, respectively (normal adult male range, LH: 1-5 and FSH: 2-9 mU/ml). Urinary androstanediol glucuronide excretion was 74 and 50μg/24 h in patients 8044 and 8047, respectively (normal adult male level, 193 ± 77μg/24 h). Both patients were castrated and treated with estro-progestative replacement therapy. Skin biopsies were obtained with informed consent at the time of surgery including genital skin (labia majora) for patient 8044 who also had a vaginoplasty, and pubic skin for patient 8047.

Androgen-binding capacity was not detectable in either patient after regular 1-hour incubation or prolonged incubations (Table 1).

However, 5α-reductase activity was normal, 68±6 fmol/μg DNA/h, in genital skin from patient 8044 (normal value, >2.1 fmol/μg DNA/h). In pubic skin fibroblasts from patient 8047, 5α-reductase activity was low, 2.5 fmol/μg DNA/h (normal male value, 3-8 fmol/μg DNA/h). These data confirmed that 5α-reductase is neither androgen dependent in genital skin nor involved in the mechanism of androgen insensitivity in these patients.

Northern blot analysis of the expression of the AR mRNA in both patients exhibited a 10-kilobase band characteristic of the normal size of the AR mRNA. This result eliminates the possibility that a gross deletion or gene rearrangement occurred (6). To further examine if there was any mutation within the AR cDNA, we cloned and sequenced the entire AR cDNA (Fig. 1).Complementary DNAs were generated by reverse transcription (RT) from total RNA isolated from skin fibroblasts of both CAI patients, and amplified by the PCR using three pairs of primers (5 and G, 2 and 4, as well as A and B) that flank the entire AR cDNA (6, 7). The amplified DNA fragments corresponding to the N-terminal, DNA-, and ligand-binding domains were then subcloned into either pCR1000 or pT7Blue plasmid, and sequenced.

Surprisingly, no mutation was found in the entire coding sequence of the AR gene from either patient (1, 15).

These results reveal an unusual insight into the AR action at the molecular level of androgen resistance. In recent years, well-defined mutations in the AR gene have been identified in patients with androgen insensitivity syndrome (3-7). However, some evidence suggested that mutations of the AR gene could be responsible for most, but not all, cases of AIS (16). Interestingly, the data described here show no mutation in the coding region of the AR gene in two siblings (8044 and 8047) with AIS. To our knowledge thus far, this may represent the first case of a normal wild-type coding sequence within the AR gene in patients with AIS. These results may, therefore, highlight the importance of an unidentified mechanism for the androgen action, apart from the AR coding region itself. Thus, translational or post-translational control required for the expression of functional AR may be a contributing factor in AIS. In addition, it could be that cells of these patients contain an inhibitor that could bind to AR and prevent hormone binding, DNA binding, and/or receptor activation. Alternatively, other AR accessory proteins (co-activators or co-factors) may be needed for the fulfillment of normal AR function in these patients (17, 18). Therefore, defects at the translational level or of accessory factors of the AR may increase the complexity of the molecular heterogeneity in this clinical spectrum.

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