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L712V mutation in the androgen receptor gene causes complete androgen insensitivity syndrome due to severe loss of androgen function

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ABSTRACT

Inability to respond to the circulating androgens is named as androgen insensitivity syndrome (AIS). Mutations in the androgen receptor (AR) gene are the most common cause of AIS. A cause and effect relationship between some of these mutations and the AIS phenotype has been proven by *in vitro* studies. Several other mutations have been identified, but need to be functionally validated for pathogenicity. Screening of the AR mutations upon presumptive diagnosis of AIS is recommended. We analyzed a case of complete androgen insensitivity syndrome (CAIS) for mutations in the *AR* gene. Sequencing of the entire coding region revealed C > G mutation (CTT–GTT) at codon 712 (position according to the NCBI database) in exon 4 of the gene, resulting in replacement of leucine with valine in the ligand-binding domain of the AR protein. No incidence of this mutation was observed in 230 normal male individuals analyzed for comparison. *In vitro* androgen binding and transactivation assays using mutant clone showed approximately 71% loss of ligand binding and about 76% loss of transactivation function. We conclude that CAIS in this individual was due to L712V substitution in the androgen receptor protein.

1. Introduction

Male secondary sexual differentiation begins during prenatal stage under the influence of androgens (testosterone and dihydrotestosterone). The message is conveyed through the androgen receptor (AR), such that receptor–testosterone complex signals the differentiation of Wolffian duct during embryonic life, regulates the secretion of luteinizing hormone by hypothalamic-pituitary axis and spermatogenesis. The more active form of testosterone, dihydrotestosterone, also acts via AR promoting the development of external genitalia and prostate during embryogenesis and male pubertal changes [1]. This crucial receptor for male development is encoded by the *AR* gene mapped onto the long arm (Xq11–12) of the X-chromosome [2]. A member of nuclear receptor super-family, AR protein has a domain organization consisting of N-terminal domain (NTD), DNA binding domain (DBD) and ligand binding domain (LBD). In addition to ligand binding, LBD also serves a role in receptor dimerization, interaction with other proteins, and nuclear localization [3].

Defects in androgen action may compromise secondary sexual development in 46,XY individuals, resulting in androgen insensitivity syndrome (AIS) (MIM# 300068). The most common genetic defects detected in AIS cases are the androgen receptor (AR) mutations, and the terms are dictated by the extent of loss of androgen function. Complete androgen insensitivity syndrome (CAIS) is characterized by female external genitalia, often with small labia, a short blind ending vagina, absent Wolffian duct derived structures and prostate, presence of gynecomastia to different degrees, and scanty pubic and axillary hair [4]. Partial androgen insensitivity syndrome (PAIS) may be represented by an array of phenotypes, with most severe form represented by predominantly female phenotype (female external genitalia with partially to completely fused labia, presence of pubic hair with or without clitoromegaly), and less severe forms represented by ambiguous genitalia to predominantly male phenotype with micropenis, perineal hypospadias, and cryptorchidism [5]. The latter group of patients is also termed as Reifenstein syndrome (MIM# 312300). PAIS patients are assigned a grade depending upon the degree of similarity to the male or female pattern. Individuals with mildest form of androgen insensitivity (MAIS) are not easy to distinguish from their normal counterparts except that during puberty they may have breast enlargement, sparse facial

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and body hair, and a relatively small penis [6]. In some MAIS cases, hypospermatogenesis may be seen [7].

Mutations in the AR gene have explained the etiology of AIS in a large number of individuals. This has not only advanced understanding of androgen action but also provided very useful information for drug development. So far, several mutations have been identified in the *AR* gene in AIS patients with different degrees of insensitivity [8] (Web URL: http://androgendb.mcgill.ca/). In the present study, we have analyzed the *AR* gene and identified a novel mutation in a case of complete androgen insensitivity syndrome.

2. Materials and methods

2.1. Subject and clinical history

The subject was recruited in the study through the Institute of Reproductive Medicine (IRM), Kolkata, India. The patient approached the clinic with the primary complaint of failure to attain menarche even up to the age of 28 years. A detailed history of the patient along with physical examination was taken, revealing well-developed breasts, female external genitals, blind ending vagina and no pubic or axillary hair (Fig. 1A). Pelvic ultrasound showed no development of Wolffian duct derived structures, well distended urinary bladder, abdominal gonads and no uterus, consistent with complete androgen insensitivity syndrome [9]. Cyst formation was seen in both the gonads. The patient had a positive family history with both the siblings affected. Both the sibs had well developed breasts, were typically feminized, and had phenotypic features consistent with CAIS. Peripheral blood sample of the patient was collected for endocrinological, cytogenetic and molecular genetic analyses. We could not get the sample of other two affected sisters and mother. The study was approved by the Institutional Ethics Committee (IEC) of the CCMB.

2.2. Hormone assays

We measured circulating levels of testosterone, leutinizing hormone (LH), and follicle stimulating hormone (FSH) in the proband using radioimmunoassays. The hormone levels were periodically measured for three consecutive weeks, and an average of three readings was taken for further considerations.

2.3. Cytogenetic analysis

Cytogenetic analysis was done as detailed in our previous study [10].

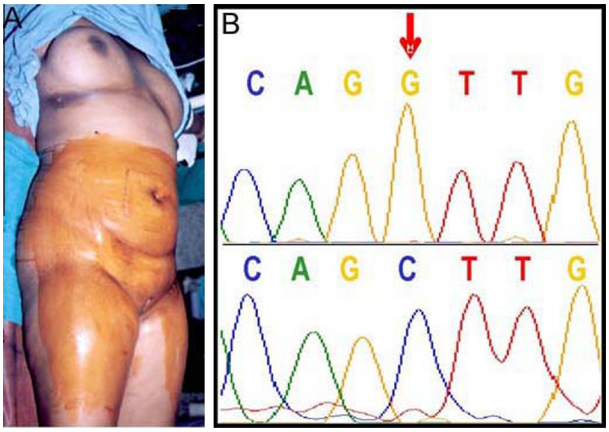

Fig. 1. (A) Phenotypic features of the patient. (B) Electropherogram showing mutation in the *AR* gene. The electropherogram for the patient is placed above that of control.

2.4. Histological studies

Abdominal gonads of the patient were removed due to cancer risk, and the tissue biopsy was subjected to histological analyses as detailed in our earlier study [10].

2.5. DNA sequencing

We extracted high molecular weight DNA from peripheral lymphocytes using phenol–chloroform precipitation method [11]. The exonic regions along with exon–intron boundaries were amplified by PCR using the sets of primers described in our earlier study [10]. Nucleotide sequence of the PCR products was read using dideoxy chain terminator cycle sequencing protocol (BigDye™) [12] and ABI 3730 DNA Analyzer (Applied Biosystems, USA). First exon, being large in size, was amplified with four overlapping pairs of primers. The number of CAG repeats was calculated as mentioned in our earlier study [11]. The target site was also analyzed in 230 normal male samples for comparison. Auto-Assembler software (Applied Biosystems, USA) was employed for DNA sequence alignment and identification of sequence variations.

2.6. Construction of mutant AR clone

The *AR* clone (pSVARo) and the reporter gene clone (pMMTV-Luc) were kind gifts from Dr. Bruce Gottlieb. Mutants were generated following the protocol published in our previous study. The desired mutation was introduced in the clone by site directed mutagenesis. We designed complementary primers bearing desired mutation in the middle of forward and reverse primers. The primers used were forward AACTGGGAGAGAGACAGGTTGTACACGTGGTCAAGT and reverse ACTTGACCACGTGTACAACCTGTCTCTCTCCCAGTT (the mutated nucleotide is highlighted in the primer sequences). The mutagenesis reaction was carried out as detailed in our earlier study [4]. 25 ng of the amplified plasmid DNA was transformed into *Escherichia coli* by heat shock at 42 °C for 45 s. Transformants were screened individually for mutant colonies, which were confirmed by direct DNA sequencing of the clones as detailed in our earlier study [4]. The plasmid from the mutant colonies was isolated on a large scale using maxiprep kit (Qiagen Inc, Valencia, CA, USA).

COS-1 cells were used for *in vitro* ligand binding and transactivation assays. The cells were transfected with 800 ng of purified plasmid using 4.8 μl lipofectamine (Stratagene, La Jolla, CA, USA) for ligand binding assays. After 72 h of transfection, the cells were washed, and culture medium replaced with a medium containing 5% charcoal stripped steroid free serum. After 96 h, the cells were harvested using 0.01% trypsin–0.02% EDTA in phosphate buffered saline (PBS). The harvested cells were divided into two fractions for Western blotting and ligand binding assays. The cells were counted using a hemocytometer to adjust cell density. Equal volume of cell suspensions with the same cell density were incubated with 1.0–10 nmol of tritium labeled methyltrienolone (R1881, a synthetic androgen agonist) in the presence and absence of 1000 fold of unlabeled methyltrienolone to determine specific and non-specific ligand binding, respectively. After 2 h of incubation, the cells were washed with 2 ml PBS three times to remove any unbound ligand. The cells were lysed with lysis solution [50 mM Tris–Cl (pH 8.0), 10 mM EDTA (pH 8.0), 100 mM NaCl, 0.5% (v/v) Triton X-100], 0.01 ml of protease inhibitor mixture (Sigma, St. Louis, MO, USA) to release intracellular contents. The whole cell lysate was then mixed with 6 ml of scintillation counting fluid Bio-Safe II (Research Products International Corporation, IL, USA) and disintegrations per second were counted with liquid scintillation analyzer (1500 TRI-CARB, Packard). The results were expressed as binding sites per 10^5 cells. The second fraction of the harvested cells was