Journal of Steroid Biochemistry & Molecular Biology 122 (2010) 359–363

Contents lists available at ScienceDirect

Journal of Steroid Biochemistry and Molecular Biology

journal homepage: www.elsevier.com/locate/jsbmb

Short communication

C601S mutation in the androgen receptor results in partial loss of androgen function☆

Rajender Singh[a,b], Pooja Singh[b], Nalini J. Gupta[c], Baidyanath Chakrabarty[c], Lalji Singh[a], Kumarasamy Thangaraj[a,*]

[a] Centre for Cellular and Molecular Biology, Uppal Road, Hyderabad 500 007, India
[b] Endocrinology Division, Central Drug Research Institute, India
[c] Institute of Reproductive Medicine, Salt Lake, Kolkata, India

ARTICLE INFO

Article history:
Received 21 April 2010
Received in revised form 12 May 2010
Accepted 12 May 2010

Keywords:
Androgen insensitivity syndrome
Androgen receptor
Sex reversal
XY female

ABSTRACT

The present study was undertaken on a case of partial androgen insensitivity syndrome to look at the etiology of the disorder. The patient exhibited a female phenotype despite 46,XY chromosome complement. Direct DNA sequencing of coding region of the androgen receptor gene in this case revealed a 2329G>C substitution (cDNA sequence reference) in exon 3 of the gene. The substitution resulted in replacement of Cys with Ser at codon 601 of the ligand-binding domain of the protein. Analyses on 200 control samples revealed absence of this substitution(s). *In vitro* assays were done using COS-1 cells. The mutation resulted in partial (~40%) loss of ligand-binding and significant (~70%) loss of downstream transactivation function. The mutation was absent in the controls. The findings are particularly interesting since another substitution at the same codon (TGC–TTC) has been reported in association with complete androgen insensitivity syndrome.

1. Introduction

Androgens (testosterone and dihydrotestosterone) initiate the process of secondary sexual differentiation during prenatal stage. The message is conveyed through androgen receptor (AR), such that receptor–testosterone complex signals the differentiation of Wolffian duct during embryonic life, regulation of secretion of leutinizing hormone by hypothalamic–pituitary axis and spermatogenesis, and receptor–dihydrotestosterone complex promotes the development of external genitalia and prostate during embryogenesis and is also responsible for changes, which occur at puberty in males [1]. Androgen receptor is encoded by the *AR* gene mapped onto the long arm (Xq11-12) of X-chromosome [2]. Eight exons of the gene encode a protein with 919 amino acid residues. 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 is also involved in nuclear localization, receptor dimerization and interaction with other proteins [3].

The end organ resistance to androgens is named as androgen insensitivity syndrome (AIS) (MIM# 300068). AIS is a very rare disorder with a frequency of approximately 1 in 30,000 live births [4]. Mutations in the *AR* gene are the most common cause of AIS. The phenotype in androgen insensitivity depends on the extent of loss of androgen function. Individuals with complete androgen insensitivity syndrome (CAIS) have female external genitalia, usually with small labial folds, a short blind ending vagina, absent Wolffian duct derived structures and prostate, gynecomastia, scanty pubic and axillary hair [5]. In partial androgen insensitivity syndrome (PAIS), several different phenotypes are evident, with predominantly female phenotype (female external genitalia, pubic hairs with or without clitoromegaly and partially to completely fused labia) in most severe form, ambiguous genitalia to predominantly male phenotype with micropenis, perineal hypospadias and cryptorchidism in less severe forms [6]. The later group of patients is also termed as Reifenstein syndrome (MIM# 312300). PAIS patients are assigned a grade according to the severity of androgen insensitivity and affinity of the phenotype with male or female pattern. Individuals with mildest form of androgen insensitivity (MAIS) usually have normal male genitals and internal male structures, and during puberty may have breast enlargement, sparse facial and body hair, and small penis [7]. Some affected individuals who otherwise are normal male may also have impaired sperm production resulting in oligozoospermia or azoospermia [8].

So far, more than 500 mutations have been reported in the *AR* gene in various grades of AIS [9]. A substitution at codon 601 (TGC–TTC) has been reported in a case of complete androgen insen-

☆ S.R., N.J.G., L.S. and K.T. have nothing to disclose.

* Corresponding author. Tel.: +91 40 27192636; fax: +91 40 27160591.
E-mail address: thangs@ccmb.res.in (K. Thangaraj).

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doi:10.1016/j.jsbmb.2010.05.006

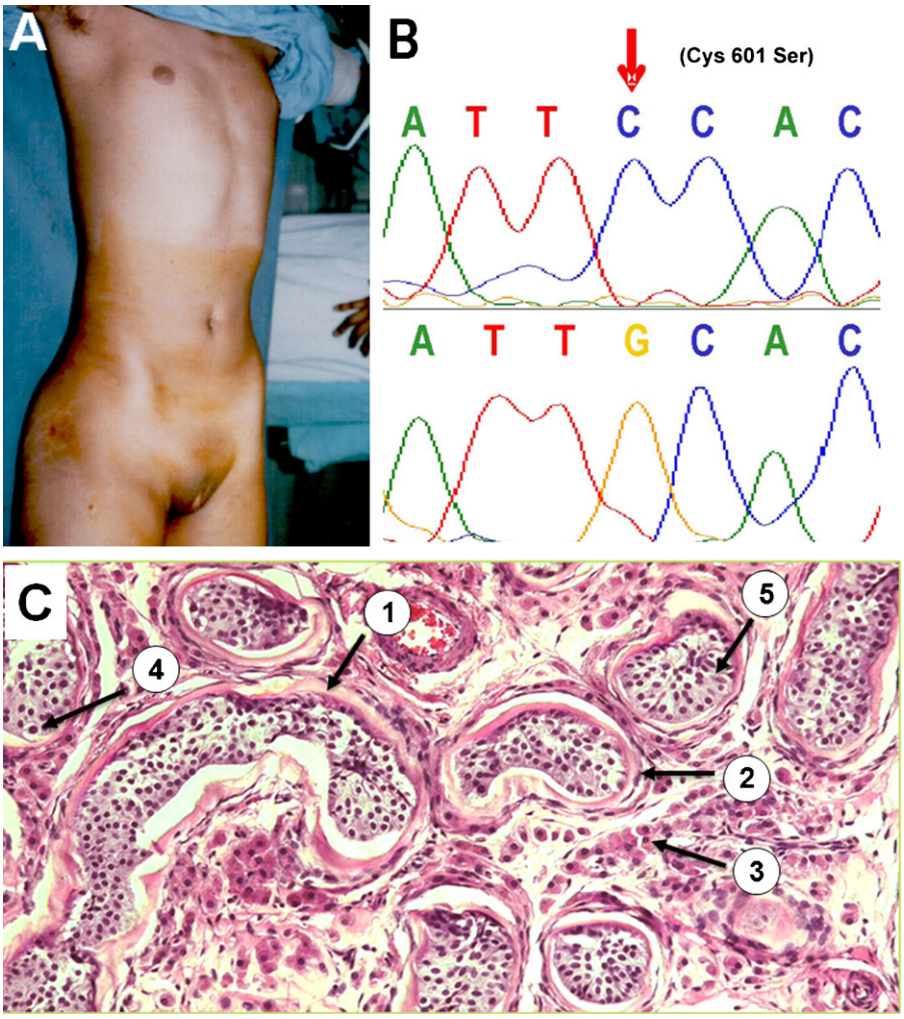

Fig. 1. Phenotypic, histological and genetic evaluation of the patient. (A) Phenotypic features showing ambiguous genitalia. (B) Electropherogram showing the mutation in *AR* gene. The electropherogram for the patient is placed above the control. (C) Histology of gonad sections (400×) showing well differentiated seminiferous tubules with thick basement membrane (1) surrounded by fibroblasts (2), interstitial space filled with Leydig cells (3), spermatogonium (4) spotted in the seminiferous tubules having lumen filled with Sertoli cells (5).

sitivity syndrome [10]; however, the functional consequences of the mutation have not been analyzed. We undertook the present study to look at the etiology of the 46,XY disorder of sexual development with partial androgen insensitivity syndrome.

2. Materials and methods

2.1. Subjects and clinical history

The subject was recruited through the Institute of Reproductive Medicine (IRM), Kolkata, India, in January 2006. The patient approached the clinic with primary complaint of absence of menarche at the age of 16 years. A detailed history of the patient was taken along with physical examination. Physical examination revealed poorly developed breasts, ambiguous genitalia with a small (1 cm) phallus/penis like structure between the partially developed labial folds, separate vaginal and urethral openings, normal pubic and axillary hair (Fig. 1A). Ultrasound followed by laparoscopic surgery of the pelvic region revealed rudimentary uterus, no fallopian tubes, normal and well distended urinary bladder, abdominal gonads and no mass lesion in pelvis. Testosterone level was in upper normal range at 23.9 nmol/L (reference: 15.15–24.51); LH and FSH were elevated at 49.60 mIU/L (reference: 7–24 mIU/L) and 50.2 mIU/L (reference: 4–25 mIU/L), respectively. Androgen insensitivity index (product of absolute values of T and LH) was also higher at 1185.44 than normal range (reference: 106.05–588.24). The above characteristics of the patient were consistent with partial androgen insensitivity phenotype [5]. The patient had one normal sister and reportedly no family history of AIS; however, mother's DNA could not be analyzed due to unavailability of the sample. Peripheral blood sample of the patient was collected for cytogenetic and molecular genetic analyses. A total 200 normal healthy male individuals were recruited as controls for the study. The study was approved by the Institutional Ethics Committee of the CCMB.

2.2. Cytogenetic analyses

Cytogenetic analysis was done as detailed in our earlier study [11].

2.3. Histological studies

Abdominal gonads of the patient were removed because of cancer risk, and the tissue biopsy subjected to histologic analyses as detailed in our earlier study [11].

2.4. DNA sequencing

DNA was extracted from peripheral blood by the protocol described in our earlier study [12]. Complete coding region of the *AR* gene along with exon–intron boundaries was amplified using primers described in our earlier study [11]. The amplicons were directly sequenced using dideoxy chain terminator cycle sequencing protocol (BigDye™) [13] and ABI 3730 DNA Analyzer (Applied Biosystems, USA). First exon, being large in size, was amplified with four overlapping pairs of primers. Multiple sequence alignment, editing and consensus sequences were built using Auto-Assembler software.

2.5. Ligand-binding assays

The *AR* (pSVARo) and reporter clone (pMMTV-Luc) were kind gifts from Dr. Bruce Gottlieb. The mutation was introduced in the AR clone by site-directed mutagenesis with QuikChange® Site-Directed Mutagenesis Kit (Stratagene, La Jolla, CA, USA), using the conditions provided by the manufacturers. The primers used were forward GCGCCAGCAGAAATGATTCCACTATTGATAAATTCCG and reverse CGGAATTTATCAATAGTGGAATCATTTCTGCTGGCGC (the mutated nucleotide is highlighted in the primer sequences). The mutated base in the primer sequence is underlined. The primers were purified by polyacrylamide gel electrophoresis (PAGE) for site-directed mutagenesis. The amplified product from site-directed mutagenesis reaction was used for bacterial transformation. Transformed colonies were picked up and plasmid was isolated using mini prep kit (Bangalore Genei, India). The mutation in the clone was checked by direct DNA sequencing of the desired region. The plasmid from the mutant colonies was isolated on a large scale using maxiprep kit (Qiagen Inc., Valencia, CA).

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-binging assays. The cells were counted using a hemocytometer to adjust cell density. Equal volumes of cell suspensions with same cell density were incubated with 0.5 nmol–2.0 nmol of methyltrienolone (R1881) in the presence and absence of 1000-fold of unlabeled methyltrienolone to determine specific and non-specific ligand binding, respectively. After 2 h incubation, the cells were washed with 2 ml PBS three times to remove any unbound ligand. The cells were lysed with cell 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, and 0.01 ml of protease inhibitor mixture (Sigma)/ml]. 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 processed for Western blotting according to the protocol described in our earlier study [14].

2.6. Transactivation assays

The cells were plated at a density of 2×10^5 in six well plates. Upon 70% confluence stage, the cells were transfected with 300 ng of the mutant or normal *AR* clones along with 70 ng of β-gal and 430 ng of MMTV-Luc plasmids using the protocol described above. After 24 h, the medium was replaced with a medium containing 5% charcoal stripped serum followed by addition of the ligand (at 0.5 nmol–2.0 nmol concentrations) to the medium. After 72 h, the cells were harvested by trypsinization and centrifugation. Upon washing with PBS, the cells were counted by hemocytometer and adjusted for uniform cell density. The cells were lysed using cell 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, and 0.01 ml of protease inhibitor mixture (Sigma)/ml]. The activity of co-transfected β-gal was measured using β-gal assay kit (Roche, Palo Alto, CA, USA) to estimate the transfection efficiency. The luciferase activity was measured by luciferase assay system (Promega Corporation, Madison, WI, USA) using TD-20/20 luminometer (Turner Design, Sunnyvale, CA, USA). Transfection efficiency was corrected by the ratio of luciferase to β-gal activity.

2.7. Comparison of AR protein sequence across different genera

The sequence of AR protein for different genera was aligned using CLUSTAL W multiple alignment program and compared to look at the variations and conservation in the region containing Cys 601 amino acid.

3. Results

3.1. C601S mutation identified in exon 3 of the AR gene

Sequencing of the entire coding region of the *AR* gene revealed 2329G>C mutation (cDNA sequence reference) (Fig. 1B). The mutation resulted in replacement of cysteine with serine at codon 601 in the DNA-binding domain of the AR protein. The mutation was absent in all the control samples analyzed.

3.2. Cytogenetic and histologic analysis

The patient had 46,XY karyotype with no apparent numerical or structural chromosomal abnormality. Histologic analysis revealed testicular tissue on both sides. The gonads had well-differentiated seminiferous tubules with thick basement membrane, but no sperm differentiation was observed (Fig. 1C). Spermatogonia could be spotted, but no indication of onset of sperm differentiation was obvious. Therefore, spermatogenesis was probably arrested at the very first step of spermatogenesis onset. Sertoli cells could be easily spotted in the seminiferous tubules. The interstitial space was crowded with Leydig cells, indicating Leydig cell hyperplasia.

3.3. Ligand-binding and transactivation function assays

The mutation was successfully incorporated in the *AR* clone, as evidenced by direct DNA sequencing of the clone in forward and reverse directions. Protein isolation, followed by Western blotting and hybridization, confirmed the expression of both wild type and mutant proteins. As expected, the mutant protein was normal in size and quantitatively similar expression to that of wild type was observed in the mutant clone. *In vitro* assays showed that the mutation resulted in approximately 40% loss of ligand-binding and approximately 70% loss of transactivation function (Fig. 2).

The saturation in ligand binding in the mutant version was observed at relatively lower concentration of the ligand (15 fmol) in comparison to the wild type (23 fMol) (Fig. 2 left panel). In the transactivation assays, the luciferase activity did not go beyond 100 even when the ligand concentration was as high as 1.5 nM (Fig. 2 right panel). The androgen function could not be rescued at high ligand concentrations as well.

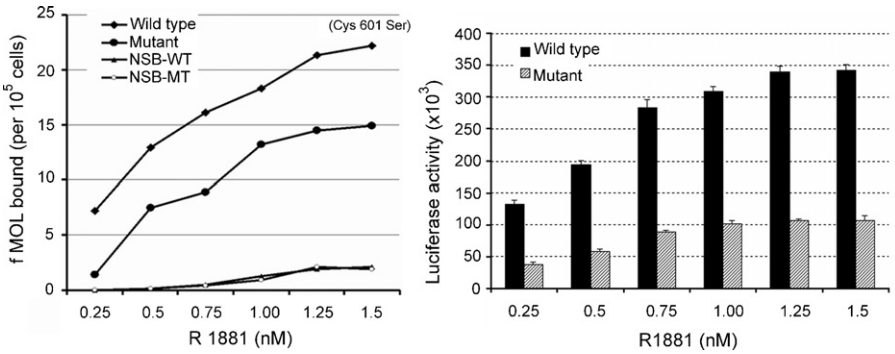

Fig. 2. Ligand-binding and transactivation assays on AR-C601S mutant. Ligand-binding assay (left panel) showing partial loss of ligand binding. NSB-WT indicates non-specific binding with wild type allele and NSB-MT indicates non-specific binding with mutant allele. Transactivation function assay (right panel) shows significant reduction of downstream transactivation function.

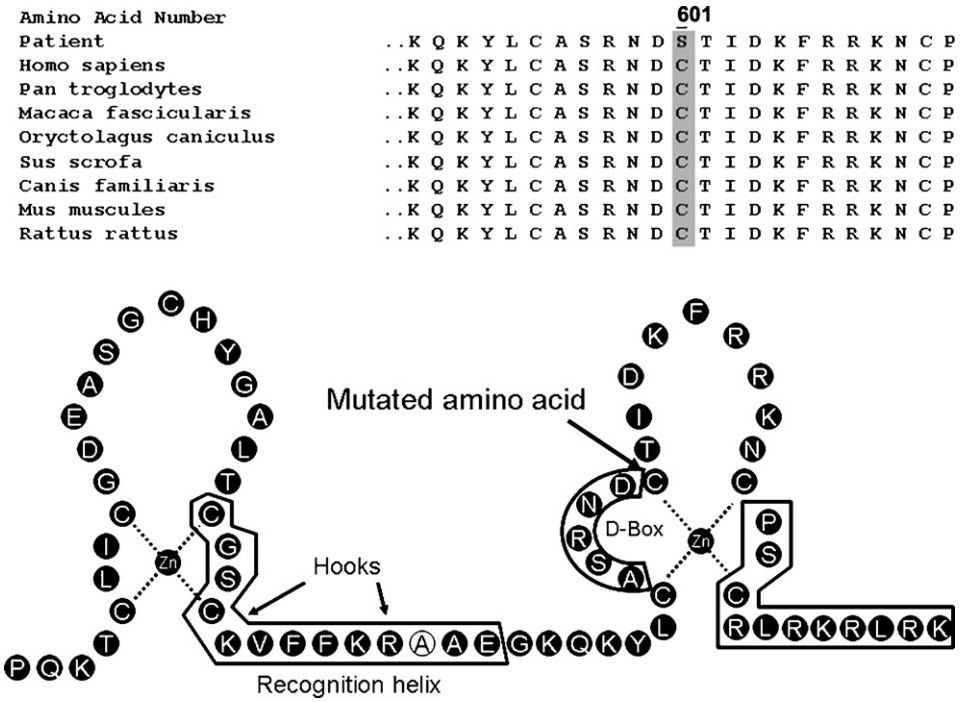

Fig. 3. Conservation and position of the mutated amino acid in the AR protein. Cysteine is highly conserved residue across mammalian species (upper panel). The mutated amino acid residue is a part of zinc finger in the AR-DBD (lower panel).

3.4. Cys 601 is a highly conserved amino acid

The alignment of AR sequences across genera showed that Cys at position 601 is a highly conserved amino acid in the androgen receptor protein. Also the stretch of amino acids on either side of this residue is highly conserved (Fig. 3 upper panel).

4. Discussion

Hundreds of mutations in the *AR* gene have been reported in association with several different disorders such as androgen insensitivity syndrome, male infertility and prostate cancer [9]. In certain instances, same mutation has been associated with different disorders such as male infertility and prostate cancer, though androgens are thought to play opposite roles in the above disorders. Not only this, same mutation has been occasionally reported in association with partial androgen insensitivity, complete androgen insensitivity and in normal individuals as well [9]. Functional assays provide very useful information under such conditions, which not only provide insights into the mechanism of androgen action but also in the role of androgens in these disorders. In our analyses in this case of partial androgen insensitivity, we observed C601S mutation in the AR gene, while the only sibling (46,XX normal female) of this patient had normal phenotype and wild type nucleotide at this position. Negative family history of AIS indicated recent origin of this mutation. The presence of a rudimentary uterus cannot be explained on the basis of existing knowledge.

This substitution (TGC–TCC) has not been reported earlier; however, another substitution of the same nucleotide with a different nucleotide (TGC–-TTC) has been reported in a case of CAIS [10]. The findings are particularly interesting since the phenotype was compromised to a milder extent in our case in comparison to the earlier report by Baldazzi et al. [10]. In the earlier report, a polar amino acid (cys) was substituted with a non-polar, aromatic amino acid (phe). In the present case, the residual AR function could be the result of fewer disturbances in the ionic environment of the DBD due to substitution of a polar amino acid (Cys) with a similar amino acid (Ser).

Cys 601 is part of a stretch of highly conserved amino acid residues in the AR-DBD (Fig. 3 upper panel), indicating its functional

significance. Partial loss of both, ligand-binding and transactivation function, despite its location in the DBD, indicated that the mutation disturbed not only the DBD but also conformation of the LBD. The crystal structure of rat AR-DBD shows two zinc-finger modules, each of which is stabilized by coordinated binding of a zinc atom by four cysteine residues [15]. Cys 601 lies in one of the zinc fingers at such a crucial site that it makes contact with zinc atom (Fig. 3 lower panel). The substituted amino acid (ser) is not equally good for interaction with zinc atom such that it may distort the finger and hence DNA binding.

The AR protein normally forms complex with the androgens and localizes to the nucleus, where it interacts with several co-activators to ultimately transcribe its message. Partial loss of ligand binding to the receptor results in the cytoplasmic localization of the free receptor with peri-nuclear distribution, enforcing its faster degradation [16]. Further, the D-box in the second zinc finger is involved in protein–protein interactions with a second receptor molecule in the homo-dimer complex. The relative positioning of the two zinc fingers must be crucial for AR function. The disturbances in the structure of zinc finger might distort the structure of the D-box such that the interaction of AR protein with co-activators in the mutant is sub-optimal.

Identification of this mutation may have important clinical and pharmacological values. In addition to understanding the structural–functional correlation of AR, *in vitro* studies, like the one here, would help in designing the molecules, which could bind to the mutated receptors and restore androgen action, and also to develop male contraceptive strategies. The abdominal gonads of the patient were removed and female external genitalia constructed. This is the first study describing this mutation and its functional consequences in AIS. More reports and functional analysis on this mutation may provide further insights into the pathogenesis of this mutation.

Acknowledgements

We are indebted to the patient and the family members for cooperation during the study. The study was supported by financial grants from the CSIR, Government of India.

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