

# L859F Mutation in Androgen Receptor Gene Results in Complete Loss of Androgen Binding to the Receptor

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**ABSTRACT:** Androgens drive male secondary sexual differentiation and maturation. Mutations in the androgen receptor (*AR*) gene cause an array of abnormal sex differentiation phenotypes in humans, ranging from mild through partial to complete androgen insensitivity. Earlier, we reported a C3693T missense mutation in the *AR* gene in a familial case of complete androgen insensitivity syndrome (CAIS), resulting in the replacement of a highly conserved leucine residue with phenylalanine (L859F) in ligand-binding domain (LBD) of the receptor. In silico analysis and the information from the crystal structure of AR-LBD indicated that the residue L859, located in helix 10 of AR protein, plays a significant role in overall architecture of the ligand-binding pocket. From this information we anticipated that the mutation might

have resulted in the loss of the ligand binding to the receptor. In the present study, we have conducted the in vitro functional assays for this mutation. The mutation resulted in highly significant loss of the ligand binding to the receptor. The loss of ligand binding and subsequent AR function was confirmed by the transactivation assay, in which we observed very little activation of the reporter gene expressed under the control of the ligand-AR complex.

**Key words:** XY sex primary amenorrhea reversal, primary amenorrhea, androgens, complete androgen insensitivity syndrome, Leydig cell hyperplasia, ligand binding.

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Androgens drive male secondary sexual differentiation and maturation. Two main androgens in humans, testosterone (T) and dihydrotestosterone (DHT), complex with the same receptor for their action but confer different biological messages. The receptor-T complex signals differentiation of the wolffian duct during embryonic life, regulation of secretion of leutinizing hormone by the hypothalamic-pituitary axis, and spermatogenesis. The receptor-DHT complex promotes development of external genitalia and prostate during embryogenesis and is also responsible for the changes that occur at puberty in males (Haqq and Donahoe, 1998).

The *AR* gene mapped to Xq11.2-q12 encodes a protein with 919 amino acids. The AR protein has a domain organization consisting of N-terminal domain, DNA-binding domain, and a C-terminal ligand-binding domain (LBD). In addition to ligand binding, LBD is also involved in nuclear localization, receptor dimerization, and interaction with other proteins (Brinkmann et al, 1989). Mutations in the *AR* gene are known to cause complete androgen insensitivity syndrome (CAIS) rep-

resented by female phenotype, partial androgen insensitivity syndrome (PAIS), with phenotype ranging from predominantly male to female, and mild androgen insensitivity syndrome (MAIS) characterized by under-masculinization (Tsukada et al, 1994) or infertility in otherwise healthy males (Rajender et al, 2007).

Hundreds of mutations have been reported in the *AR* gene worldwide (Gottlieb et al, 2004). Most of these studies are accompanied by functional assays to show the mechanism of the pathogenesis of the mutation. Various mutations in the *AR* gene have resulted either in the loss of androgen binding to the receptor molecule or loss in the transactivation potential of the ligand-AR complex without significant loss in the ligand binding. In our earlier report, we identified a C3693T (mRNA position) missense mutation in the *AR* gene in a familial case of CAIS. On the basis of the information from the nature of the mutation and its location in the ligand-binding pocket of the receptor molecule, we proposed that the mutation might have resulted in the loss of the ligand binding (Singh et al, 2006). We have now conducted in vitro functional assays to prove the mechanism of action of the mutation.

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## **Subjects, Materials, and Methods**

### *Subjects and Clinical History*

The details of the subjects and the clinical record have been provided in our earlier study (Singh et al, 2006). All the

affected individuals in the family matched the clinical features of CAIS. Direct DNA sequencing for the proband and other affected individuals in the family resulted in the identification of L859F mutation. The segregation of the mutation with the disorder in this family indicated this mutation to underlie the disorder. However, the functional significance of this mutation in the pathogenesis of CAIS has now been elucidated by the ligand binding and transactivation assays. This study was approved by institutional ethical committee at CCMB.

#### Construction of the Mutant AR Clone

The AR clone (pSVARo) and the reporter clone (pMMTV-Luc) were the kind gifts from Dr Bruce Gottlieb. We designed the complimentary primers having the mutation in the middle of forward and reverse primers. The 2 primer sequences were as follows: forward: 5' GACGCTTCTACCAGTTACCAAGCTCCTG 3' (the mutated base is underlined); reverse: 5' CAGGAGCTTGGTGAACTGGTAGAAGCGTC 3' (the mutated base is underlined). The whole plasmid molecule bearing the AR clone was amplified using QuikChange site-directed mutagenesis kit (Stratagene, La Jolla, Calif) with the primers bearing the desired mutation. The 50- $\mu$ L polymerase chain reaction (PCR) consisted of PCR buffer 5.0  $\mu$ L, each primer 50 pmol, plasmid DNA 50 ng, deoxy nucleotide tri phosphates (dNTPs) 1.0  $\mu$ L, and *PfuTurbo* DNA polymerase 1.0  $\mu$ L. The reaction was set up under the following PCR conditions: 95°C for 30 seconds followed by 16 cycles of denaturation at 95°C for 30 seconds, annealing at 55°C for 1 minute, and polymerization at 68°C for 10 minutes with a final extension at 68°C for 10 minutes. The amplicon from the above reaction was incubated at 37°C for 1 hour with *DpnI* restriction enzyme to digest the wild-type (parental) plasmid molecules. Upon digestion, the amplified product was checked on 1% agarose gel for quantitative and qualitative analyses.

#### Bacterial Transformation and Plasmid Isolation (Miniprep)

The amplified product was used for the transformation in *Escherichia coli*; 25 ng of the plasmid DNA was transformed into *E coli* by heat shock at 42°C for 45 seconds. The ampicillin-resistant colonies were picked up and used for plasmid isolation. The plasmid DNA was isolated with the Mini prep kit (Bangalore Genei, India) following the protocol provided by the manufacturers. The DNA was dissolved in an appropriate quantity of tris-EDTA (TE) buffer. After incubating at room temperature for 2 hours, the plasmid DNA was checked on 2% agarose gel for qualitative and quantitative evaluation.

#### Direct DNA Sequencing and Plasmid Isolation (Maxiprep)

The plasmid DNA (approximately 400 ng) was directly sequenced to confirm the presence of the mutation in the clone using Big-Dye chain terminator cycle sequencing protocol on 3730 DNA analyzer (Applied Biosystems, Foster City, Calif) (Thangaraj et al, 2003). The plasmid was isolated on a large scale using the maxiprep kit (Qiagen Inc, Valencia, Calif) from the mutant colonies following the protocol provided by the manufacturers. The plasmid DNA was evaluated on 2% agarose gel for quantitative and qualitative analyses.

#### Androgen-Binding Assay

About 800 ng of the plasmid DNA was used for transfection of COS1 cells using 4.8  $\mu$ L of lipofectamine (Stratagene, La Jolla, Calif). After 72 hours of the transfection, the cells were washed with PBS and medium replaced with a medium containing 5% charcoal-stripped steroid-free serum. After 96 hours, the cells were harvested using 0.01% trypsin–0.02% EDTA in phosphate-buffered saline (PBS). The harvested cells were divided into 2 fractions for Western blotting and ligand-binding assays. The cells were counted by using a hemocytometer to adjust the cell density. Equal volumes of cell suspension with same cell density were incubated with 0.5–2.0 nmol of methyltrienolone (R1881) in the presence and absence of 1000-fold unlabeled methyltrienolone to determine specific and nonspecific ligand binding, respectively. After the incubation, the cells were washed with 2 mL of PBS 3 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% [vol/vol] Triton X-100, and 0.01 mL of protease inhibitor mixture per mL [Sigma-Aldrich Corporation, St Louis, Mo]). The whole cell lysate was then mixed with 6 mL of scintillation counting fluid Bio-Safe II (Research Products International Corp, Mount Prospect, Ill), and the disintegrations per second were counted with the liquid scintillation analyzer (1500 TRI-CARB; Packard, Downers Grove, Ill). The results were expressed as binding sites per  $10^5$  cells.

#### Western Blotting

The harvested cells were lysed in the Laemmli buffer (50 mM Tris with pH 6.8, 2% sodium dodecyl sulfate [SDS], 10% glycerol, 5%  $\beta$ -mercaptoethanol, 0.01% bromophenol blue) and boiled for 10 minutes in a water bath according to the method of Laemmli (1970). The cell lysate was centrifuged at 10 000 rpm for 3 minutes (Biofuge pico, Heraeus Instruments, Hanau, Germany) and supernatant collected. Protein content of each lysate was estimated in the supernatant by Amido Black method (Kaplan and Pedersen, 1985). Equal amounts of the protein for different samples were loaded on SDS–polyacrylamide gel electrophoresis (SDS-PAGE) (10%) and electrophoresed at 80 V in running buffer (25 mM Tris, 250 mM glycine, 1% SDS with pH 8.3). The resolved proteins were electrophoretically transferred to a nitrocellulose membrane (Hybond C, Amersham Life Sciences, Little Chalfont, United Kingdom) following the semidry method of Towbin et al (1979). Subsequently the membrane was stained with 0.1% Ponceau S (prepared in 1% acetic acid) to check the efficiency of the transfer. Before hybridization with the antibodies, the membrane was washed with distilled water to remove the Ponceau stain and blocked with 5% (wt/vol) nonfat milk in TBST (150 mM NaCl, 20 mM Tris-HCl, 0.1% Tween 20, pH 7.6) for 2 hours at room temperature. Subsequently, the membrane was washed and incubated with 1:10 000 dilution of primary antibody in TBST. After 2 hours of incubation with the primary antibody, the membrane was washed with TBST (3 times, 5 minutes each) and incubated with 1:1000 dilution of alkaline phosphatase–conjugated secondary antibody in TBST. After 1 hour of incubation, the membrane was washed with TBST (3 times, 5 minutes each). Upon hybridization, the blot

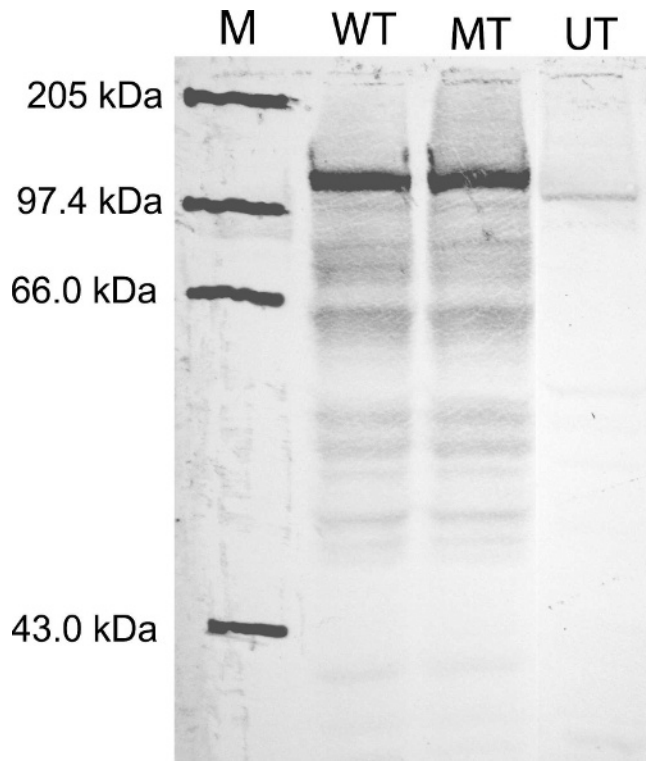


Figure 1. Western blotting. Western blotting showed normal expression of the androgen receptor (AR) protein in the cells transfected with wild-type and mutant clones.

was incubated in alkaline phosphatase buffer (100 mM NaCl, 5 mM MgCl<sub>2</sub>, 100 mM Tris, pH 9.5) to which nitro-blue tetrazolium chloride (NBT) (66  $\mu$ L for 10 mL of buffer) and 5-bromo-4-chloro-3'-indolylphosphate p-toluidine (BCIP) salt (33  $\mu$ L for 10 mL buffer) were added. The blot was incubated in the dark until a distinct band appeared.

#### Transactivation Assays

The cells were plated at a density of  $2 \times 10^5$  in 6-well plates. Upon 70% confluency stage, the cells were transfected with 300 ng of the mutant or normal AR clones along with 70 ng of  $\beta$ -galactosidase ( $\beta$ -gal) and 430 ng of MMTV-Luc plasmids using the protocol described above. After 24 hours, the medium was replaced with a medium containing 5% charcoal-stripped serum followed by the addition of the ligand (0.5–2.0 nmol) to the medium. After 72 hours, the cells were harvested by trypsinization and centrifugation. After washing with PBS, the cells were counted by hemocytometer and adjusted for the 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% [vol/vol] Triton X-100, and 0.01 mL of protease inhibitor mixture per mL [Sigma]). The activity of the cotransfected  $\beta$ -gal assay plasmid was measured using  $\beta$ -gal assay kit (Roche, Palo Alto, Calif) to estimate the transfection efficiency. The luciferase activity was measured by a luciferase assay system (Promega Corp, Madison, Wis) using a TD-20/20 luminometer (Turner Design, Sunnyvale, Calif).

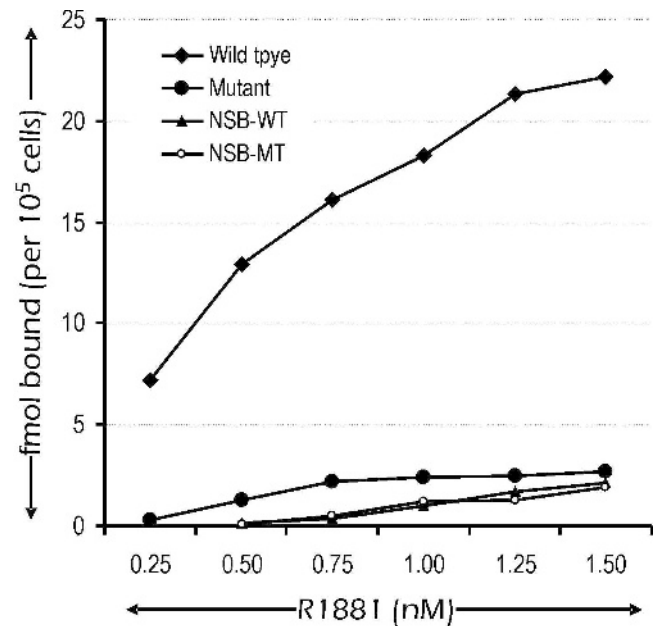


Figure 2. Ligand-binding assay. The ligand-binding assay showed a drastic decrease in the binding of R1881 to the mutant receptor in comparison with the wild-type receptor. Radiolabeled R1881 and unlabeled R1881 were used to determine the specific and non-specific ligand binding, respectively. nonspecific binding-wild type (NSB-WT) shows the nonspecific ligand binding to the wild-type receptor, and nonspecific binding-mutant type (NSB-MT) shows the nonspecific binding to the mutant receptor.

Transfection efficiency was corrected by the ratio of the luciferase activity to  $\beta$ -gal activity.

## Results

Site-directed mutagenesis resulted in the successful incorporation of the mutation C3693T in AR clone. The presence of the mutation in the clone was confirmed by its sequencing in forward and reverse directions. Protein isolation followed by Western blotting and hybridization confirmed the expression of both the wild-type and the mutant proteins (Figure 1). Ligand-binding assay showed negligible binding of R1881 to the mutant receptor in comparison with the wild type under all concentrations of the ligand used (Figure 2). Complementing the results of binding assay, the transactivation assay showed very less activation of the reporter gene in the mutant receptor in comparison with the wild-type receptor (Figure 3).

## Discussion

Our earlier study on a familial case of CAIS revealed C3693T mutation in exon 7 of the AR gene, leading to

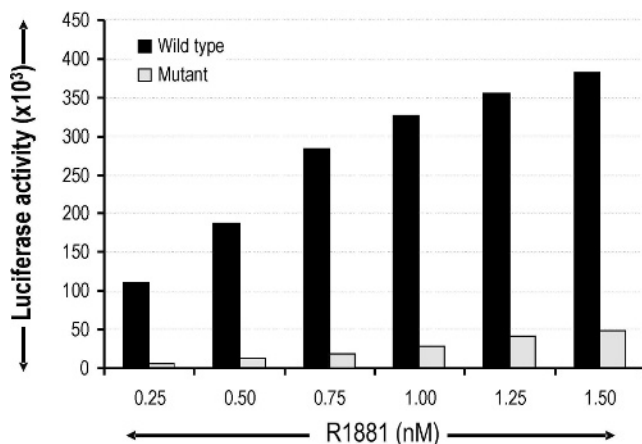


Figure 3. Transactivation assay. Transactivation assay was done to check the activity of the ligand-receptor complex in the wild-type and mutant receptors. The activity of the luciferase enzyme expressed under the control of ligand-androgen receptor complex responsive promoter was measured as a function of transactivation. The assay showed negligible transactivation in the mutant receptor in comparison with the wild-type receptor.

replacement of leucine with phenylalanine at codon 859 of AR (Singh et al, 2006). L859 is a part of AR-LBD, and highly conserved across diverse species (Singh et al, 2006). The importance of this amino acid residue in AR function is further strengthened by its critical location in the ligand-binding pocket of the receptor molecule. In the previous study, we hypothesized that the ligand-binding pocket in the mutant receptor is unable to attain proper conformation, resulting in total disruption of ligand binding and hence CAIS. In the present study, we have functionally proved that it is the highly significant loss of ligand binding that resulted in the disorder in this family.

The *in vitro* assays revealed that the androgen binding was reduced to negligible levels as a result of the mutation. This could be due to the fact that the mutated amino acid localized to LBD. However, the observation of no transactivation in the mutant receptor must be due to the loss of complex formation between androgen and AR protein. The AR protein normally forms complex with the androgen and localizes to the nucleus. However, the absence of ligand or failure of ligand binding to the receptor results in the cytoplasmic localization of the receptor with perinuclear distribution, enforcing its faster degradation (Kemppainen et al, 1992). Therefore, the mutations resulting in complete loss of androgen binding should result in failure of nuclear localization of the AR protein, resulting in rapid degradation. Similarly, the complete loss of androgen binding as a result of L859F mutation in the present case would have rendered the AR protein nonfunctional along with its rapid degradation as a result of cytoplasmic localization.

Most of the reported mutations associated with androgen insensitivity syndrome localize to LBD and are available at the androgen receptor mutation database along with resulting phenotypes (Gottlieb et al, 2004). The mechanism of pathogenesis of most of these mutations is well supported by the functional assays. The mutations resulting in the replacement of the amino acids by similar other amino acids should result in partial loss of function, whereas the replacement of an amino acid by a dissimilar amino acid should result in complete loss of function. However, this did not hold true when we compared the nature of amino acid substitution with the extent of androgen insensitivity for the mutations reported in exon 7 to date (available at the AR mutation database) (Gottlieb et al, 2004). Exon 7 is the second highly mutated region of the AR gene after exon 5. More than 37 different types of mutations have been reported in 127 individuals (Gottlieb et al, 2004). Of these mutations, the most (20 mutations) were reported in CAIS cases, followed by PAIS (11 mutations), CAIS and PAIS (4 mutations), MAIS (1 mutation), and prostate cancer (1 mutation). Many mutations replacing a highly dissimilar amino acid resulted in PAIS and did not alter the ligand binding, whereas many other mutations replacing a similar amino acid without significantly altering ligand binding resulted in CAIS. Likewise, the mutation under study resulted in the replacement of a hydrophobic amino acid with another hydrophobic amino acid but resulted in CAIS. However, unlike other mutations, we observed complete loss of ligand binding. The above discussion shows that the ultimate phenotype in AR mutations does not depend upon the kind of amino acids replaced but on the extent of the conservation of that particular amino acid and its location in the 3-dimensional crystal structure of the protein.

Mutations in the AR gene are known to show phenotypic variation in different individuals. Substantial variations have been noted in the familial cases bearing the same mutation. Somatic mutations in the androgen target tissues have been proposed to contribute to phenotypic variation. However, somatic mutations have been able to explain the phenomenon only in a few cases of the more than 25 cases of phenotypic variation reported to date (Gottlieb et al, 2001a,b). All the patients in the present family exhibited same phenotype; however, the phenotypic variation associated with this mutation cannot be ruled out in further generations if somatic mutations in the androgen-responsive tissues result in the back mutation. Certain mutations in the AR gene are even known to result in a gradient of the phenotypes in different affected individuals. However, this is the first report of the L859F mutation; therefore, phenotypic variations asso-

ciated with this mutation have to wait for more studies reporting the same mutation.

The patients were given estrogen therapy and underwent surgery to construct the female genital organs. We are in communication with the patients to provide genetic counseling for future pregnancies of their healthy siblings to abandon the transmission of the mutated X chromosome to the coming generations.

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