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# A Novel Androgen Receptor Mutation Resulting in Complete Androgen Insensitivity Syndrome and Bilateral Leydig Cell Hyperplasia

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**ABSTRACT:** Androgens drive male secondary sexual differentiation and maturation. Mutations in the androgen receptor (*AR*) gene cause a broad spectrum of abnormal phenotypes in humans, ranging from mild through partial to complete androgen insensitivity. We have analyzed the *AR* gene by using denaturing high-performance liquid chromatography (DHPLC) and direct sequencing and have studied gonads histologically in a familial case of complete androgen insensitivity syndrome. Sequence analysis of the *AR* gene showed a novel C2578T missense mutation, resulting in the replacement of a highly conserved leucine residue with phenylalanine (L859F) in ligand-binding domain of the receptor. The residue L859, located in helix 10 of the androgen receptor, plays a significant role in overall architecture of ligand-binding pocket. The mutation was absent from the father, normal brother of the patients, and 100 normal males recruited in this study as controls. The inheritance of the mutation in the family clearly shows that C2578T is the underlying mutation for the eventual phenotype in the patients. Histology of patient's gonads showed Leydig cell hyperplasia, with a few or no spermatogonium. It is thought that *AR* gene mutations result in hormonal imbalance, resulting in the high levels of luteinizing hormone (LH) and ultimately Leydig cell hyperplasia or tumor formation. In the present study, we have reported a rare familial case of Leydig cell hyperplasia despite consistently normal LH levels. The finding will help in giving counseling to this family and prevent the transmission of the mutated X chromosome to the coming generations.

Key words: Androgens, androgen receptor gene.

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Upon testes differentiation, androgens drive male secondary sexual differentiation and maturation. For normal male sexual differentiation, 2 main androgens—testosterone (T) and dihydrotestosterone (DHT)—and a functional androgen receptor (*AR*) gene are required. Both these androgens complex with the same receptor for their action but confer different biological messages. The receptor-T complex signals differentiation of Wolffian ducts during embryonic life, regulation of secretion of luteinizing hormone (LH) by hypothalamic-pituitary axis, and spermatogenesis. The receptor-DHT complex promotes development of external genitalia and prostate gland during embryogenesis and is also responsible for the changes that occur at puberty in males (Haqq and Donahoe, 1998).

*AR* gene mapped to Xq11.2-q12 encodes a protein with 919 amino acids. AR protein has a domain organization consisting of a N-terminal domain or transactivation domain, a DNA-binding domain, and a 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 varying levels of androgen insensitivity syndrome (AIS). Mild AIS (MAIS) is characterized by undermasculinization (Tsukada et al, 1994) or infertility in otherwise normal males (Wang et al, 1998). In partial AIS (PAIS), several different phenotypes are evident, ranging from predominantly female phenotype to ambiguous genitalia or predominantly male phenotype with micropenis, perineal hypospadias, or cryptorchidism (Quigley et al, 1995). Complete AIS (CAIS) is characterized by female external genitalia usually with small labial folds, a short blind ending vagina, absence of Wolffian duct–derived structures and prostate, breast development, and scanty or absent pubic and axillary hairs.

AIS patients are at a higher risk of developing gonadal tumors. Seminomas are the most common type of tumors in these patients. Other malignancies such as Sertoli cell tumors and Leydig cell tumors are rare. We report a novel mutation in the *AR* gene resulting in

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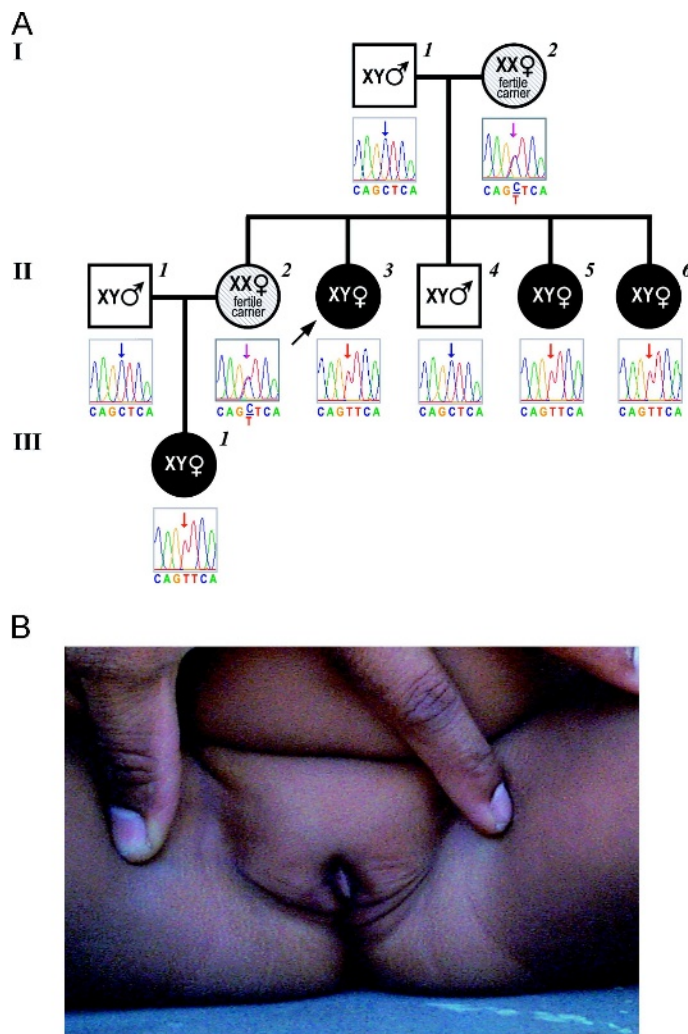


Figure 1. **(A)** Pedigree of the family with complete androgen insensitivity syndrome (CAIS) and the electropherogram showing the mutations. Individuals with CAIS have been designated by black circles. The sequence electropherogram of the androgen receptor gene is shown below. The proband (II-3) (shown by arrow) possessed the mutant allele "T" (highlighted with arrow) at the nucleotide position 3693 (base position in mRNA). Similar mutation is also seen in all the individuals with CAIS (II-5, II-6, and III-1). Both the fertile females in this pedigree (I-2 and II-2) possessed the heterozygous allele C/T, whereas all 3 males (I-1, II-1, and II-4) possessed the wild-type allele "C." **(B)** Clinical picture of individual with CAIS. Clinical picture of 2-year-old child with CAIS (III-1) shows female external genitalia with small labial folds and no fusion of labia, characteristic features of CAIS.

complete androgen insensitivity and Leydig cell hyperplasia in 3 patients within a family.

## Materials and Methods

### *Subjects and Clinical History*

The family belonged to a suburban area of Hyderabad, Andhra Pradesh, India. The proband (individual II-3 in Figure 1A) did not attain menarche up to the age of 20 years and hence approached the reproductive center of MNR Medical College Hospital, Medak, Andhra Pradesh, India. The parents (I-1 and I-2) had no complaints regarding reproduction. Upon recording the family history, it was found that 2 of the proband's siblings (II-5 and II-6) also did not attain menarche up to the age of 18 years and 16 years, respectively. A team of highly qualified clinicians carried out clinical evaluation of the subjects. Physical examination of these patients revealed typical female phenotype with female external genitalia, a short blind ending vagina, breast development, and scanty axillary and pubic hairs. Real-time ultrasonography of the pelvis showed nondevelopment of Wolffian duct–derived structures, well-distended urinary bladder with no calculi and postvoid residue, no uterus, and mass lesions in the pelvis. The patients had 1 apparently normal brother (II-4) and 1 fertile sister (II-2); the later attained puberty at the age of 13 years with normal feminine development. She married an apparently normal man and delivered a child with 46,XY karyotype, female external

genitalia having small labial folds and a blind ending vagina, characteristic of CAIS (Figure 1B). Blood samples of all the family members were collected with their informed written consent and were subjected to endocrinological, cytogenetic, and molecular analysis. Gonads of the patients (II-3 and II-5) were surgically removed because of the risk of cancer and were subjected to histological examination. This study was approved by the institutional ethical committee.

### Hormone Assays

Serum levels of T, follicle-stimulating hormone (FSH), and LH were measured by radioimmunoassays for the proband and the affected siblings. The hormone levels were periodically measured after every 7 days, and an average of the 3 readings was taken for further considerations.

### Cytogenetic Analysis

Peripheral blood lymphocyte cultures were set up in duplicate for all the family members in 5-mL culture vials with Roswell Park Memorial Institute media supplemented with 10% fetal calf serum. Cells were grown in the presence of penicillin-streptomycin-gentamycin. Phytohemagglutinin was added to stimulate cell division. Dividing cells were arrested at metaphase stage with colchicin and fixed in methanol and acetic acid (3:1). Fixed cells were dropped onto glass slides and allowed to air dry. Chromosomes were G-banded by treating the preparations with trypsin followed by staining with Giemsa.

### Histological Studies

Gonadal tissue of the proband and 1 of the affected siblings (II-5) was fixed with 10% buffered neutral formalin solution at room temperature for 7 days. Tissue was dehydrated with isopropanol, cleared with xylene at room temperature, and impregnated with paraffin wax at 58°C. Tissue embedded in paraffin wax was cut into 4-μm thick sections with a LeicaRM2135 microtome (Leica, Germany). The sections were directly taken on egg albumin–coated glass slides and kept at 60°C for 1 hour. Slides were dewaxed with xylene and stained with hematoxylin followed by eosin. After staining, slides were mounted with dipthyline xylene and observed with an Axioplan imaging system (Zieman, Zeiss, Germany). Images were captured at different magnifications.

### Denaturing High-Performance Liquid Chromatography and DNA Sequence Analysis

DNA was extracted from peripheral blood samples of all the family members and controls by using a protocol described elsewhere (Thangaraj et al, 2002). Polymerase chain reaction (PCR) primers for the *AR* gene were taken from our earlier study (Singh et al, 2006). Eleven pairs of primers amplified all the exons of the *AR* gene including the exon-intron splice junctions. The 10-μL PCR reaction mixture included 1.0 μL PCR buffer (10X), 1.0 μL $MgCl_2$ (25 mM), 0.8 μL deoxy nucleotide tri-phosphates (10 mM), 0.5 pM of each primer, 1 unit of AmpliTaq Gold DNA polymerase (Applied Biosystems, Foster City, Calif), and 20 ng of genomic DNA. PCR conditions for exon 7 consisted of initial denaturation at 94°C for 12 minutes followed by 30 cycles of denaturation at 94°C for 45 seconds, annealing at 58°C for 45 seconds, and extension at 72°C for 1 minute, with a final extension at 72°C for 10 minutes.

Amplicons of all coding regions of *AR* gene of the patients, controls, and both mixed were subjected to denaturing high-performance liquid chromatography (DHPLC) analysis. The optimum oven temperature for heteroduplex separation was selected from the melting profile of the amplicon. After denaturation and gradual annealing (0.1°C/min) of PCR product, DHPLC was performed by using the WAVE nucleic acid fragment analysis system (Transgenomics Inc, Omaha, Neb). The column was given an equilibration run with buffer B (0.1 mM TEAA, 25% vol/vol acetonitrile) before injection of the sample. Aliquots of 8 μL PCR product were loaded on a preheated C18 reverse phase column based on polystyrene-divinyl benzene particles. DNA was eluted from the column by a linear acetonitrile gradient in 0.1 mM triethylamine acetate buffer (TEAA), pH 7.0, at a constant flow rate of 1.5 mL/min at 58°C. The gradient was formed by mixing buffer A (0.1 mM TEAA) and buffer B. Elution of DNA was detected by ultraviolet absorbance at 260 nm. To confirm the mutation, PCR product of exon 7 was directly sequenced by using dideoxy chain terminator cycle sequencing protocol (BigDye V3.1, Foster City, Calif ) (Thangaraj et al, 2003) on 3700 DNA analyzer (Applied Biosystems). Later, all the remaining exons of the *AR* gene were directly sequenced to confirm absence of any other mutation in the gene.

## Results

### Hormone Levels

The proband and 2 siblings tested were found to have T levels slightly elevated: 670–710 ng/dL (normal male range 437–707 ng/dL). Levels of FSH and LH were within normal range at 4.29–4.59 mIU/mL (normal male range 4–25 mIU/mL) and 15.29–16.21 mIU/mL (normal male value 7–24 mIU/ml), respectively.

### Cytogenetic Analysis

Karyotype of all the 4 individuals with CAIS revealed 46,XY complement. None of the patients or family members showed structural or numerical chromosomal abnormality.

### Histological

Hematoxylin-eosin–stained 4-μm paraffin sections of the gonads showed widely dispersed seminiferous tubules with thickened basement membrane. The tubules were lined by about 3–5 layers of cells with elongated cytoplasmic outlines and condensed darkly stained round to oval nuclei. Some of the tubules showed occasionally large flatter cells with large round nuclei having open chromatin resembling spermatogonium, but no cell

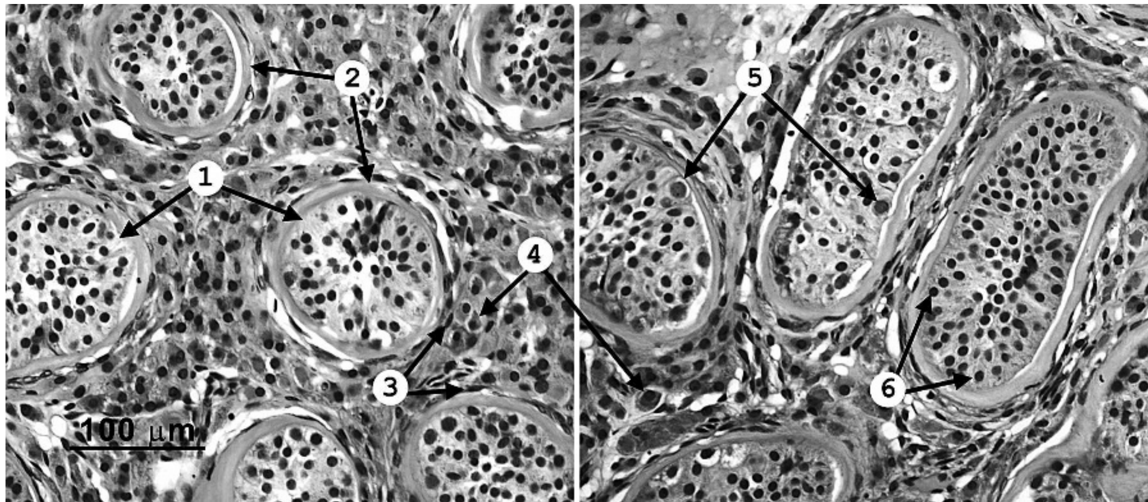


Figure 2. Transverse section of the gonad of the proband. Gonadal section of the proband (II-3) showed hypoplastic testis containing widely dispersed seminiferous tubules (1) with thick basement membrane (2) surrounded by fibroblasts (3). The interstitial space contains excess number of Leydig cells (4), known as Leydig cell hyperplasia. The seminiferous tubules occasionally contain spermatogonium (5) but totally filled with Sertoli cells (6).

division could be seen in these cells. All other cells in seminiferous tubules confirmed to Sertoli cell morphology, which filled up the whole of the lumen of seminiferous tubule. The size of the seminiferous tubule lumen was comparatively smaller, giving the appearance of a highly dense Sertoli cells population. The interstitial space was filled with darkly stained eosinophilic granular cytoplasm and single round nuclei, consistent with Leydig cells. Overall, it appeared to be an immature testis, mostly with Leydig cell hyperplasia (Figure 2).

### DHPLC Analysis

DHPLC analysis indicated a mutation in exon 7 of the *AR* gene of all the patients. Normal males (I-1, II-1, and II-4) possessed wild-type allele (Figure 3A), individuals with CAIS (II-3, II-5, II-6, and III-1) possessed mutant allele (Figure 3B), and carrier females (I-2 and II-2) showed heterozygous pattern (Figure 3C). Mixture of amplicons of normal male (I-1) and individual with CAIS (II-3) showed the same pattern as heterozygous (Figure 3D).

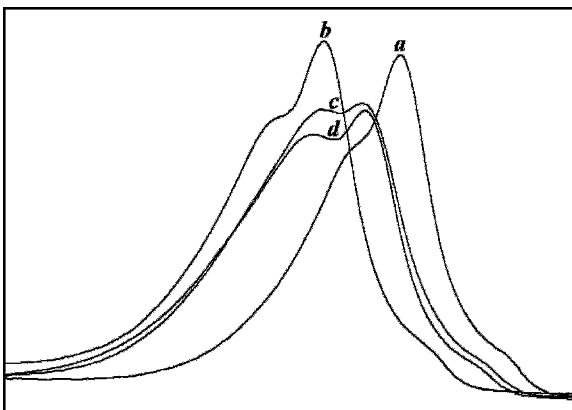


Figure 3. Denaturing high-performance liquid chromatography analysis of family with complete androgen insensitivity syndrome. **(A)** Wild-type allele (I-1, II-1, and II-4 in Figure 1). **(B)** Mutant allele (II-3, II-5, II-6, and III-1). **(C)** Heterozygous allele (I-2 and II-2). **(D)** Mixture of wild and mutant alleles.

### Identification of the Mutation

Sequence analysis showed that all the patients had C2578T hemizygous mutation in exon 7 of the *AR* gene resulting in the replacement of leucine with phenylalanine (L859F) in the LBD of the receptor molecule. The father (I-1), an apparently normal brother (II-4), and individual II-1 had wild-type allele (C), whereas the mother (I-2) and a fertile sister (II-2) possessed heterozygous allele (C/T) at nucleotide position 3693 (Figure 1A). As is evident from Figure 1A, the mutation was transmitted to the second and third generations in similar fashion. The mutation was confirmed by sequencing PCR products from 3 independent reactions both in forward and reverse directions. Sequence analysis of all other *AR* exons confirmed normal sequence. None of the 100 control samples analyzed showed mutation at this nucleotide position.

## Discussion

The *AR* gene has been well studied in androgen insensitivity and a number of mutations have been reported, resulting in various grades of androgen insensitivity in genetically male individuals. Although

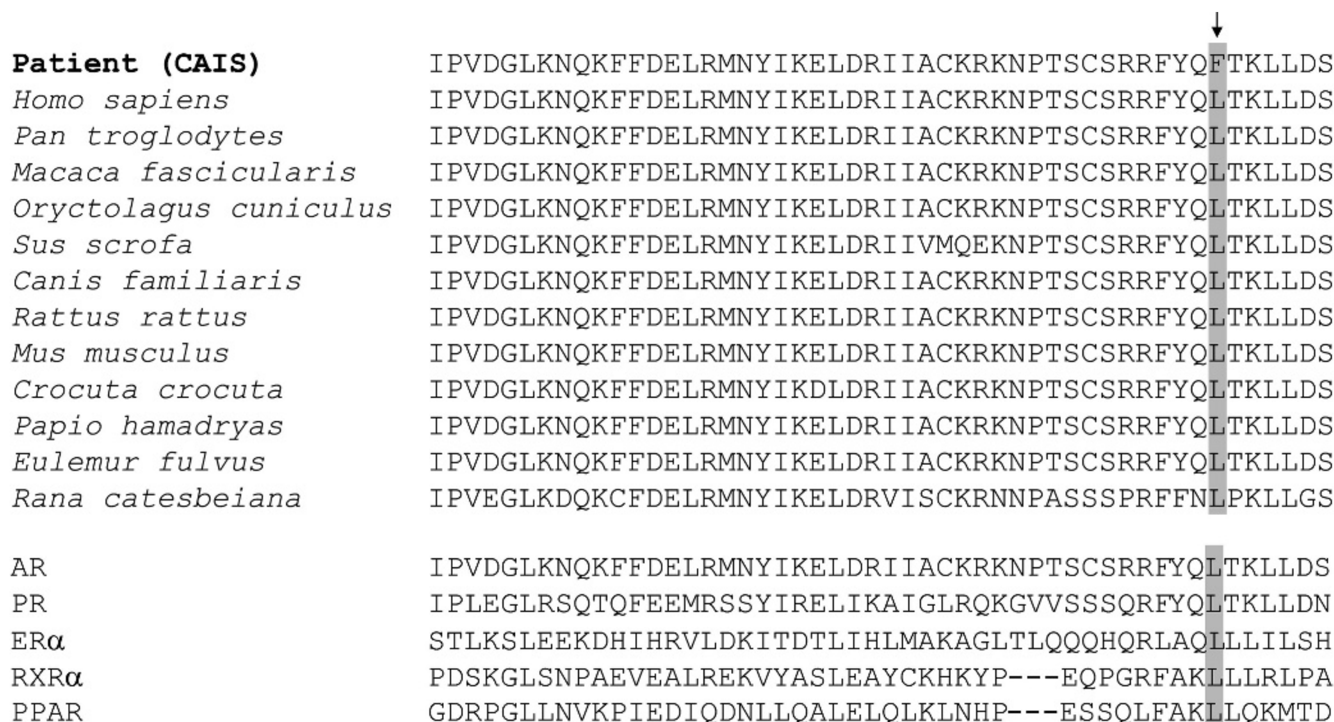


Figure 4. Multiple alignment of androgen receptor (AR) protein sequence. Multiple alignment of AR protein across species (upper panel) and among steroid receptor super family members (lower panel) shows high conservation of L859 residue (highlighted by gray bar).

several mutations have been reported in each exon of the gene, most of the reported mutations associated with AIS localize to LBD. All the reported mutations in the *AR* gene are available at the androgen receptor mutation database along with resulting phenotypes (Gottlieb et al, 2004) (website: http://www.mcgill.ca/androgendb).

Our present study on a familial case of CAIS revealed a novel C2578T mutation leading to replacement of leucine with phenylalanine at codon 859 (exon 7 of the gene) of AR. Amino acid residue L859 is a part of LBD of AR molecule. This residue is highly conserved, both across species (Figure 4, upper panel) and in distantly related receptors (members of steroid receptor super family) such as progesterone receptor, estrogen receptor alpha, retinoid X receptor alpha, and peroxisome proliferator-activated receptor gamma (Figure 4, lower panel).

The importance of this amino acid residue in the functioning of the androgen receptor is further strengthened by 3-dimensional (3-D) architecture of the LBD. Elucidation of 3-D crystallographic structure of AR-LBD has established 12 α helices and 4 β strands arranged in 2 β sheets, which make a typical helical sandwich to form a ligand-binding pocket (Matias et al, 2000). The 12 helices of the AR protein are folded into a 3-layered sandwich. Helices H1/2, H3, H7, and H10/11 form 2 outer layers, whereas inner layers consist of a ligand-binding pocket and a non–ligand-binding hydrophobic core (helices H4/5, H8, and H9). M807 in the middle of H8 makes vanderwall interactions with I799 and F794 in H7, L859, and L863 in H10 from outer layers of sandwich and with adjacent F747 in H5 and L838 in H9 of the hydrophobic core.

Furthermore, the atomic distances between residues corresponding to M807 in H8 and L859 and L863 in H10 in steroid receptor super family are similar and highly conserved. The conservation of key residues and atomic distances in H8 and H10 support that M807 (H8)-L859 (H10) interactions are critical for the stability of holo-LBD (Ong et al, 2002). We suspect that mutation of one of the key residues L859 in helix 10 in the present case might be resulting in the disruption of the critical interactions between these conserved residues. The ligand-binding pocket in the mutant receptor is probably unable to attain proper conformation, resulting in total disruption of ligand binding and hence leading to the complete androgen insensitivity.

Similar to the L859F mutation in the present study, most of the mutations falling in the α-helical and β sheet regions of the receptor result in CAIS and those falling in the "turns" and "linker" regions result in PAIS;

however, a general correlation is difficult to derive because some mutations in the helices and β sheet regions cause PAIS and those in the turns and linker regions cause CAIS (Yong et al, 1998). Interestingly, the same mutation in different patients has been reported to cause different levels of androgen insensitivity (Gottlieb et al, 2004). G2445A mutation is known to cause both PAIS and CAIS. More interestingly, C2296A mutation, with proven pathogenicity in PAIS and CAIS patients, was also found to be present in a totally normal person, and G995A mutation known to cause MAIS, PAIS, and CAIS has also been reported to exist in 8% of the normal population (Gottlieb et al, 2004) (website: http://www.androgendb.mcgill.ca/). In the present study, sequencing of the exon 7 of the *AR* gene in 100 control samples showed complete absence of this mutation in the normal population.

In AIS patients, seminomas are the most common type of testicular tumors. Very few reports are available on adenomas and hyperplasia. Leydig cell hyperplasia or tumors are rare tumors of male gonadal interstitium that comprise approximately 3% of testicular neoplasmas. Rutgers and Scully (1991) reported the first case of Leydig cell tumor in a patient with AIS. The present study represents such a rare case of bilateral Leydig cell hyperplasia. The patients underwent gonadectomy at the ages of 18 years and 20 years. Generally, the risk of developing testicular neoplasia is 3.6% by the age of 25 years and 33% by the age of 50 years. Given hyperplasia at an age of 20 years, these patients were highly likely to develop malignant tumors in the later stages of life.

Despite many studies on AIS, it is still unclear what exactly determines the type of gonadal cell undergoing hyperplasia or tumor formation. Because many studies refer to hyperplasia or tumor of only 1 gonad (Iwamoto et al, 2005), another confounding issue is to decipher the factors responsible for selective malignancy of 1 particular gonad in certain cases. It has been reported that consistently high levels of LH result in Leydig cell tumors formation (Fort et al, 1995). Surprisingly, in the present case, Leydig cell hyperplasia developed despite normal levels of LH. A better understanding of the hormonal regulation of Sertoli and Leydig cell activity and spermatogenesis will help in understanding the correlation between the nature of *AR* gene mutation and the type of cell undergoing hyperplasia or tumor formation. Somatic mutations in the androgen target tissues may be another factor affecting the preferential hyper proliferation of certain types of testicular cells.

To conclude, the inheritance of the mutation through generations in this family shows that the abnormality could be attributed to the mutation reported here. It is one of the rare cases of bilateral Leydig cell hyperplasia. Our finding would be helpful in providing counseling to this family and possibly helping them get a normal male or female child by prenatal diagnosis, hence the transmission of the mutated X chromosome to the coming generations can be prevented.

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