# Genetic Findings in a Cohort of Patients with Androgen Insensitivity Syndrome

**Wencui Wang**
Shanghai Jiao Tong University Medical School Affiliated Ruijin Hospital
**Sichang Zheng**
Shanghai Jiao Tong University Medical School Affiliated Ruijin Hospital
**Yu Zhao**
Shanghai Institute of Endocrine and Metabolic Disease
**Zhihan Wan**
Shanghai Jiao Tong University Medical School Affiliated Ruijin Hospital
**Rulai Han**
Shanghai Institute of Endocrine and Metabolic Disease
**Yan Qi**
Shanghai Jiao Tong University Medical School Affiliated Ruijin Hospital
**Guang Ning**
Shanghai Institute of Endocrine and Metabolic Disease
**Shouyue Sun**
Shanghai Jiao Tong University Medical School Affiliated Ruijin Hospital
**Lei Ye** ( lei_yelei@163.com )
Shanghai Jiao Tong University Medical School Affiliated Ruijin Hospital https://orcid.org/0000-0003-4064-4460
**Weiqing Wang**
Shanghai Jiao Tong University Medical School Affiliated Ruijin Hospital

**Research**

**Keywords:** androgen insensitivity syndrome, AR, molecular diagnosis, transcription activity, nuclear localization

**Posted Date:** May 21st, 2021

**DOI:** https://doi.org/10.21203/rs.3.rs-499273/v1

## Abstract

**Background**: Androgen insensitivity syndrome (AIS) is a rare X-linked recessive inherited disorder caused by mutations in *AR*, a gene encoding androgen receptor. The aim of this study was to expand genetic spectrum of AIS.

**Methods:** We performed a retrospective study on consecutive patients diagnosed as AIS from 2010 to 2020 in a single tertiary center. Variant analysis of *AR* gene was performed by PCR-Sanger sequencing. The pathogenicity of novel variants was evaluated by dual-luciferase reporter assay and immunofluorescence of AR protein in vitro.

**Results:** A total of 19 unrelated 46,XY patients were enrolled, 14 with complete insensitivity syndrome (CAIS) and 5 with partial insensitivity syndrome (PAIS). We identified 19 *AR* variants: 12 (63.2%) were missense variants and 7 variants (36.8%) resulted in premature stop codon. Eight *AR* variants were novel, including P15Afs*69, S258Efs*47, W435Gfs*44, C560F, C577W, C580Afs*46, K718X and V819G. Dual-luciferase reporter assay found residual transcription activity of approximately 1% in six novel variants, which may explain the CAIS phenotype. The AR mutant protein (V819G) showed transcription activity of 59%, consistent with mild clinical features in one PAIS patient. Interestingly, another AR mutant protein (K718X) related to CAIS showed increased transcription activity but impaired nuclear localization.

**Conclusions:** We identified eight novel *AR* variants related to AIS. Both residual transcription activity of AR and nuclear localization of AR protein were associated with the severity of AIS.

## Background

As the receptor of androgen, *AR* is essential for androgen to exert its biological effects, including sex differentiation and gonadal development during fetal life, puberty development, spermatogenesis and bone metabolism in adults[1, 2]. The best illustration for androgen to play a role in male sex differentiation and gonadal development is androgen insensitivity. Androgen insensitivity syndrome (AIS) is an X-linked recessive disease and a disorder of androgen action which is the most common cause of 46,XY disorders of sex development (DSD)[3]. It is estimated that among live-born infants, the prevalence of 46,XY females is 6.4 per 100,000, of which androgen insensitivity is related to 4.1 per 100,000[4]. Depending on the severity of androgen resistance, AIS included complete, partial and mild AIS[5]. Patients with complete androgen insensitivity syndrome (CAIS) presented with female genitalia at birth, sparse or absent pubic hair at puberty, growth spurt, and breast development without menarche. The manifestation of partial androgen insensitivity syndrome (PAIS) and MAIS can be quite various and occult, ranging from hypospadias, bifid scrotum, cryptorchidism to micropenis. Gynecomastia was common at puberty in PAIS patients[1]. Patients with mild androgen insensitivity syndrome (MAIS) is generally associated with normal male genitalia but infertility in adults[6].

*AR* locates on X chromosome (Xq11-q12) and belongs to the nuclear receptor superfamily. After binding with androgen, AR undergoes conformational changes for dimerization and translocates from cytoplasm to nucleus, binds to androgen response element (ARE) and further activates the downstream target gene DNA expression[7]. The androgen receptor is comprised by four distinct functional domains, including N-terminal domain (NTD), the DNA-binding domain (DBD), the hinge domain and the ligand-binding domain (LBD)[8]. Missense mutations are the most common type, exceeding 60% of all *AR* variants. There is no hot spot mutation, but exons 5 and 7 with the ligand-binding domain are affected more frequently[9]. *AR* mutations have been implicated in a wide range of human diseases, including complete androgen insensitivity syndrome, hypospadias, male infertility and prostate cancer[2]. Even with the same substitution, the phenotype may be considerable heterogenous. Confirmed molecular diagnosis and assessment of transcription activity may not only expand the genetic spectrum of AIS, facilitate the clinical individualized management, also in favor of genetic counseling to avoid the birth of affected children. Otherwise, the probability of the offspring and sibling being the carrier is as high as 50%.

In the current study, we analyzed the genotype of *AR* gene in 19 unrelated patients with androgen insensitivity syndrome, and further assessed transcriptional activity of the novel *AR* variants.

## Results

# Clinical characteristics of patients with androgen insensitivity syndrome

From 2010 to 2020, there were 19 unrelated patients diagnosed as androgen insensitivity syndrome in Ruijin hospital, including 14 CAIS patients and 5 PAIS patients. Table 1 showed clinical characteristics of the 19 patients. In terms of CAIS patients, the median age at the first presentation was 14 (IQR 1.5–22) years, and the median confirmed diagnosis age was 18.5 (IQR 16–22) years. All female patients except case 7 complained about primary amenorrhea. Seven CAIS patients (7/14, 50%) visited a local hospital for inguinal swelling before puberty. Three patients (case 4, 7, 10) underwent bilateral inguinal swelling resection in early childhood. Case 1 and 14 with regular sexual life were admitted to adult endocrinology because of primary amenorrhea and infertility. All five patients with the diagnosis of partial androgen insensitivity syndrome were born with hypospadias, and three of them (case 15, 16 and 17) presented with cryptorchidism at birth. Four male patients (4/5, 80%) were referred to the pediatrics for gynecomastia at puberty. All male patients complained about varying degrees of small larynx, sharp voice, poor physical strength, easy to fatigue, and sparse axillary or pubic hair growth.

Table 1
Clinical and molecular characteristics of 19 patients with androgen insensitivity syndrome.

| No. | Type | Gender | Age (years) | Clinical presentation | Family history | Tanner stage | LH (mIU/mL) | FSH (mIU/mL) | T (ng/ml) | Exon | Nucleotide change | Amino acid change |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| 1 | CAIS | Female | 28 | right inguinal swelling primary amenorrhea | N | B5P1 | 17.18 | 4.51 | 4.57 | 1 | **c.39_40insC** | **p.Pro15Alafs*69** |
| 2 | CAIS | Female | 22 | primary amenorrhea | Y | B1P1 | 19.45 | 9.69 | 3.76 | 1 | **c.769_770insT** | **p.Ser258Glufs*4** |
| 3 | CAIS | Female | 14 | primary amenorrhea | N | UK | 15.48 | 18.47 | 3.11 | 1 | **c.1302delC** | **p.Trp435Glyfs*4** |
| 4 [a] | CAIS | Female | 18 | bilateral inguinal swelling primary amenorrhea | N | B1P1 | 17.89 | 26.93 | 0.48 | 1 | c.1443C > A | p.Tyr481Ter |
| 5 | CAIS | Female | 25 | primary amenorrhea | N | B3P1 | 15.9 | 1.92 | 6.24 | 2 | **c.1679G > T** | **p.Cys560Phe** |
| 6 | CAIS | Female | 15 | bilateral inguinal swelling primary amenorrhea | N | B5P1 | 28.56 | 9.65 | 9.39 | 2 | **c.1731T > G** | **p.Cys577Trp** |
| 7 [a] | CAIS | Female | 9 | bilateral inguinal swelling | Y | B1P1 | 3.2 | 46.17 | 0.8 | 2 | **c.1738delT** | **p.Cys580Alafs*4** |
| 8 | CAIS | Female | 22 | primary amenorrhea | N | B4P1 | 16.12 | 5.86 | 6.16 | 4 | **c.2152A > T** | **p.Lys718Ter** |
| 9 | CAIS | Female | 19 | primary amenorrhea | N | B4P2 | 31.01 | 28.1 | 8.14 | 5 | c.2197G > A | p. Asp733Asn |
| 10 [a] | CAIS | Female | 16 | bilateral inguinal swelling primary amenorrhea | N | B1P1 | 16.23 | 36.16 | 0.48 | 5 | c.2227A > G | p.Met743Val |
| 11 | CAIS | Female | 16 | left inguinal swelling primary amenorrhea | N | B2P3 | 30.77 | 17.44 | > 15 | 5 | c.2248A > G | p.Met750Val |
| 12 | CAIS | Female | 18 | bilateral inguinal swelling primary amenorrhea | N | B3P1 | 23.8 | 7.65 | 12.31 | 6 | c.2324G > A | p.Arg775His |
| 13 | CAIS | Female | 19 | primary amenorrhea | N | B4P1 | 30.47 | 8.19 | 6.14 | 6 | c.2359C > T | p.Arg787Ter |
| 14 | CAIS | Female | 26 | primary amenorrhea | N | B4P1 | 23.32 | 2.96 | 4.9 | 8 | c.2678C > T | p.Pro893Leu |
| 15 | PAIS | Male | 10 | hypospadias | N | B1P1G1 | 2.94 | 7.09 | 5.29 | 2 | c.1705G > T | p.Gly569Trp |
| 16 | PAIS | Male | 20 | hypospadias gynecomastia | UK | B2P3G3 | 20.07 | 46.77 | 2.89 | 3 | c.1789G > A | p.Ala597Thr |
| 17 | PAIS | Male | 15 | hypospadias gynecomastia | N | B3P2G3 | 9.62 | 7.56 | 11.13 | 5 | c.2246C > A | p.Ala749Asp |
| 18 | PAIS | Male | 26 | hypospadias gynecomastia | Y | UK | 18.37 | 5.75 | 19.56 | 7 | **c.2456T > G** | **p.Val819Gly** |

CAIS, complete androgen insensitivity syndrome. PAIS, partial androgen insensitivity syndrome. UK: unknown. “a”, cases with bilateral inguinal swelling resec
amino acid changes with bold font indicate novel variants. Reference range: LH (1.80-11.78 mIU/mL), FSH (3.0-8.1 mIU/mL), T (male 1.42–9.23 ng/ml, fema

| No. | Type | Gender | Age (years) | Clinical presentation | Family history | Tanner stage | LH (mIU/mL) | FSH (mIU/mL) | T (ng/ml) | Exon | Nucleotide change | Amino acid change |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| 19 | PAIS | Male | 16 | hypospadias gynecomastia | Y | B3P2G5 | 3.58 | 0.65 | 13.11 | 7 | c.2531C > A | p.Ala844Glu |

CAIS, complete androgen insensitivity syndrome. PAIS, partial androgen insensitivity syndrome. UK: unknown. “a”, cases with bilateral inguinal swelling resec amino acid changes with bold font indicate novel variants. Reference range: LH (1.80-11.78 mIU/mL), FSH (3.0-8.1 mIU/mL), T (male 1.42–9.23 ng/ml, fema

Physical examination showed the median height of 19 patients was 168 (IQR 161–170) cm and median BMI was 20.1 (IQR 17.7–22.8) kg/$m^2$. Breast development (Tanner B2-B5) occurred in nine female patients. Pubic hair was present in two female patients (Tanner P2-P3). Three male patients presented with gynecomastia with Tanner stage B2-B3. To be noted, four patients (4/18, 22.2%) each had one sibling of the same disease.

## Molecular analysis of AR gene

Germline *AR* gene variant was identified in all 19 patients (Table 1), including 12 missense variants, 3 nonsense variants, 4 frameshift variants. Eight variants were firstly reported (Fig. 1). Three novel frameshift variants (c.39_40insC, c.769_770insT, c.1302delC) resulted in premature termination codon in exon 1. Another small deletion (c.1738delT) in exon 2 was found in case 7. Case 5 carried the novel missense variant c.1679G > T which caused the amino acid at position 560 to be converted from cystine to phenylalanine (p.Cys560Phe). Case 6 was discovered with a missense variant (c.1731T > G) which resulted in cystine to tryptophan at amino acid position 577 (p.Cys577Trp). Another nucleotide substitution from A to T (c.2152A > T) was identified in case 8 produced a stop codon at amino acid 718 (p.Lys718Ter). A patient with PAIS (case 18) carried the novel missense variant c.2456T > G (p.Val819Gly).

Among 19 variants found in the study, variants in exon 1, 2 and 5 were the most common, each accounting for 21.1% (4/19). Ten out of 19 variants located on ligand-binding domain which could affect the receptor-binding ability with androgen. Five variants located on DNA-binding domain (DBD) and the rest of four variants resided in N-terminal domain, encoding mostly by exon 1 (Fig. 1).

## Transactivation assay of eight AR mutant proteins

We generated COS-1 cells transiently expressing wildtype AR and mutant AR proteins (P15Afs*69, S258Efs*47, W435Gfs*44, C560F, C577W, C580Afs*46, K718X, V819G). Dihydrotestosterone (DHT, final concentrations at 1nM/10nM/100nM) was treated in transfected COS-1 cells and the transcription activity was evaluated with dual-luciferase reporter assay. As seen in Fig. 2, with the increasing concentration of DHT, the transcription activity of wildtype AR improved gradually. Compared with wildtype AR, the six AR mutant proteins (P15Afs*69, S258Efs*47, W435Gfs*44, C560F, C577W, C580Afs*46) exhibited more than 95% reduced transcription activity with relative residual activity as 1.03 ± 0.07, 1.21 ± 0.09, 1.03 ± 0.04, 1 ± 0.19, 0.82 ± 0.16, 1.02 ± 0.3% and did not respond to DHT concentration gradient. To be noted, the AR mutant protein (V819G) showed mild AR-deficient activity with the residual activity of 59.33 ± 2.31% and responded to DHT stimulation in COS-1 cells in a dose-dependent manner. Interestingly, another AR mutant protein (K718X) identified in one CAIS patient showed increased transcription activity (242.99 ± 52.27%) compared with wildtype AR under different DHT concentrations.

## Nuclear localization of the AR mutant protein K718X

To further explore the underlying cause of CAIS phenotype in case 8, nuclear localization normality of the AR variant K718X was validated. COS-1 cells were transiently transfected with three different plasmids (pCMV-GFP-empty, pCMV-GFP-$AR_{WT}$ or pCMV-GFP-$AR_{K718X}$) separately. As seen in Fig. 3, under the stimulation of 100nM DHT, the pCMV-GFP-$AR_{WT}$ translocated into the nucleus from cytoplasm. However, the pCMV-GFP-$AR_{K718X}$ plasmid still existed in the cytoplasm and accumulated around the nucleus after DHT stimulation. Therefore, the AR mutant protein K718X resulted in impaired AR function through abnormal nucleus localization.

## Discussion

In this study we reported 19 unrelated patients with androgen insensitivity syndrome whose phenotype was in relation to the nature of the specific *AR* variant. A total of 19 *AR* variants were identified. Eight variants were firstly reported. Six novel *AR* variants showed seriously impaired transcription activity approximately of 1%. One AR mutant protein (V819G) presented mild AR-deficient activity of 59%. Interestingly, another AR mutant protein (K718X) showed increased transcription activity but impaired nuclear localization. The current study broadened the genetic spectrum of androgen insensitivity syndrome which may facilitate in the real clinical-setting for individualized medical approach.

During embryonic development, sex differentiation of male fetus required androgen secreted by Leydig cells to act on normal androgen receptor to exert its biological effects. Subsequently, the wolffian duct developed, male external genitalia differentiated and testes gradually descended to the scrotum. Phenotype spectrum of AIS patients is broad and closely related to the severity of AR impairment, ranging from typical female genitalia at birth to male hypospadias, micropenis, cryptorchidism, oligospermia and gynecomastia. In our study, half of the CAIS patients (50%) came for consultation due to inguinal swelling before puberty which is easily overlooked but an important clue for early diagnosis of AIS. Like Costagliola’s results[10], the median age of genetic diagnosis for CAIS patients in our study was delayed compared with the age of first symptom occurrence (18.5 [16–22] vs 14 [IQR 1.5–22]). It is reported that CAIS patients accounted for 1–2% in infancies presented with inguinal hernia which is rare in normal female infants, and nearly 57% of the CAIS population presented with an inguinal hernia in the U.K[11, 12]. Therefore, further examination in females with inguinal hernia, especially karyotype analysis, is needed for timely diagnosis of CAIS. Two female patients had regular sexual life without vagina creation and dilation. Although the vagina varies from dimple in the perineum to normal length, most CAIS women were satisfied with their psychosexual development and sexual function[13]. Another two CAIS patients presented pubic hair (Tanner P2-P3) which developed in sparse CAIS patients while axillary hair was reported generally absent[1, 14].

Partial androgen insensitivity syndrome should be excluded in infants presented with hypospadias. All five male patients in the current study showed varying degrees of severities of hypospadias. Urethral rupture was repaired and testicular descent was performed in three patients. The prevalence of DSD is estimated to be 5‰ in births, with 73% of them being boys with hypospadias[15]. Although success in reaching a molecular diagnosis in 46,XY DSD was relatively low, as Eggers et al reported in a large international patient cohort, *AR* presented to be the largest percent of variants identified in 46,XY DSD patients [16, 17]. For male patients with gynecomastia at puberty, the possibility of AIS should be considered. 80% (4/5) patients complained about gynecomastia in our study. Retrospective studies have reported gynecomastia often occurred in young adulthood of PAIS and MAIS patients and it was usually associated with signs of under virilization, such as hypospadias and cryptorchidism[18, 19]. To be noted, 22.2% (4/18) patients in our cohort had a sibling with AIS. Molecular studies in Touzon's study revealed other affected or carrier relatives in 87% of the index cases[20]. Therefore, genetic counseling for the individual and family should be strongly encouraged.

More than 1000 *AR* mutations have been reported so far, and up to 30% were sporadic de novo mutations[14]. Most *AR* mutations were identified in androgen insensitivity syndrome, and a small number of variants were detected in prostate cancer, spinal and bulbar muscular atrophy[21]. Missense variants in the current study accounted for the most 63.2% (12/19), followed by small insertions and deletion 21.1% (4/19) and nonsense mutations 15.8% (3/19). Seven out of 19 variants (36.8%) result in premature stop codon of the AR protein. The variant pattern was similar with the previous studies that most *AR* variants in all AIS phenotypes are non-synonymous point mutations and frameshift leading to a premature stop codon are more frequently reported in CAIS patients[22]. However, different from the previous studies which found that exon 5 and exon 7 were commonly involved exons in *AR*, most variants in our study located on exon 1, exon 2 and exon 5, each accounting for 21.1% (4/19). This may be due to the high proportion of CAIS enrolled, as Batista et al said, defects in NTD (mainly encoded by exon 1) are more frequent in CAIS patients[22]. Four variants (A749D, A844E, G569W, A597T) have been reported to be associated with partial AIS which is consistent with phenotype presentation in our study[23–26]. A novel *AR* variant V819G on ligand-binding domain was identified in case 18 with PAIS. It is reported that most missense mutations involved in LBD are related to partial AIS[27]. Ten variants (52.6%) resided in ligand-binding domain which has been reported to be the most frequently involved domain.

AR belongs to the nuclear receptor superfamily which needs to combine with androgen to form a complex and enter to nucleus, then activates transcription factors and promotes the expression of downstream genes. It is widely expressed in the body, such as liver, adipose tissue, endometrium, ovary, prostate, testis, skin, etc., and is closely related to the occurrence and development of many diseases. The residual activity of the affected AR underlies the phenotype of AIS and could be analyzed based on reporter assay in vitro. In our study, the transcription activity analysis demonstrated six out of eight AR mutant proteins (P15Afs*69, S258Efs*47, W435Gfs*44, C560F, C577W, C580Afs*46) have severely impaired residual activity in vitro (about 1%) which showed a strong correlation between genotype and phenotype. In terms of the two variants C560F and C577W which located in DNA-binding domain, different nucleotide substitution at the same position has been reported and all variants were associated with the phenotype of CAIS[28, 29]. The four frameshift variants resulting in premature stop codon have never been reported and the transcriptional activity was severely impaired. In contrast to decreased transcription activity, the AR mutant protein (K718X) on ligand-binding domain showed activated transcription compared with wildtype under DHT stimulation at different concentrations. The similar phenomenon has been reported in Bevan et al study[30]. The two variants D864N and L907F were identified in complete androgen insensitivity and presented with considerable binding and transactivation activity. Considering the mechanism of nuclear receptor action, we further studied nuclear localization function of the novel AR variant K718X. Under 100nM DHT, the AR variant K718X showed impaired transport function from the cytoplasm to nucleus in COS-1 cells whose normality is vital to exert androgen biological function. In fact, in terms of the correlation between phenotype and genotype of *AR* variant, it is heterogenous even with the same substitution. A small number of mutations, as L581R, R608Q, R609K, Q641X, L723F, R727L, W742C, W752X, Y764C, R787X, Q799E, V867L, were identified in patients with androgen insensitivity syndrome but also found to be gain of transcription activity in prostate cancer which may imply the complex mechanism of AR action [9]. The AR mutant protein (V819G) showed residual activity of 59%. The patient with the *AR* variant V819G presented with mild hypospadias and gynaecomastia at puberty. Reporter assay showed increased transcription activity with gradually elevated DHT concentration which further confirmed the proof of pathogenicity and severity. As Hellmann et al reported gynaecomastia may be ameliorated by androgen therapy, definite molecular diagnosis of AIS may reveal the severity of the *AR* variant and contribute to the individual's clinical management.

Our findings suggested the necessity of genetic test and functional study in patients with AIS, especially in PAIS patients. For female patients presented with inguinal swelling before puberty, karyotype analysis is greatly encouraged. Functional analysis of the *AR* variant may be in favor of assessment of the responsiveness to androgen treatment. For those variants with increased transcription activity, AR function may be severely affected through abnormal nuclear localization in androgen insensitivity syndrome.

## Conclusions

We reported 19 unrelated patients with AIS and identified eight novel *AR* variants. Inguinal swelling in females before puberty was a key clue for timely diagnosis of CAIS. We also confirmed the phenotype of AIS patients was closely associated with the residual AR activity, and transcription activity assessment could indicate the severity of the disease, thus provide professionals and individuals with available medical approach. Besides, our results indicated that *AR* variants could affect its normal function in different ways which may be the underlying causes of considerable heterogenous phenotypes.

## Methods

# Patients

A series of 19 patients with the diagnosis of androgen insensitivity syndrome from unrelated families during 2010 to 2020 were collected in Department of Endocrine and Metabolic Diseases, Shanghai Jiao Tong University School of Medicine, Ruijin Hospital. The study was approved by Ruijin Hospital Ethics Committee, Shanghai Jiao Tong University School of Medicine. All patients and/or their parents signed an informed consent for the genetic study. The

diagnosis of androgen insensitivity syndrome was made according to the Williams Textbook of Endocrinology 14th (including karyotype, physiologic findings, and hormone profile) and further confirmed by molecular analysis of the *AR* gene.

## Serum hormone measurements

Blood samples were collected in the morning and immediately centrifuged at 4°C. Serum testosterone (T), luteinizing hormone (LH), follicle-stimulating hormone (FSH) were measured by chemiluminescence immunoassay (Abbott Laboratories, Abbott Park, IL).

## Sequence analysis of AR gene

Genomic DNA of the subjects and their family members was isolated from peripheral leucocytes using QIAamp DNA Blood Mini Kit (Qiagen, Hilden, Germany). Eight exons and the flanking splicing site of *AR* (NM_000044.6) gene were amplified with primers. The PCR products were purified by QIAquick PCR purification kit (Qiagen, Hilden, Germany) and sequenced in both sense and antisense direction on ABI 3700 sequencer (Applied Biosystems Perkin-Elmer, Foster City, CA). The *AR* variants were interpreted according to American College of Medical Genetics and Genomics (ACMG) Standards and Guidelines, including the variant frequency in populations (1000 Genomes, Exome Aggregation Consortium, dbSNP, etc), inclusion in disease databases (Clin Var, Human Gene Mutation Database, etc), pathogenicity assessment by in silico predictive programs (SIFT, PolyPhen-2, Mutation Taster, etc), well-functional data and segregation with the disease in family members.

## Plasmid construction and cell culture

The plasmid pCMV-GFP-$AR_{WT}$ encoding wildtype androgen receptor (NM_000044.6) was constructed. Another eight novel variants (p.Pro15Alafs*69, p.Ser258Glufs*47, p.Trp435Glyfs*44, p.Cys560Phe, p.Cys577Trp, p.Cys580Alafs*46, p.Lys718Ter, p.Val819Gly) were introduced into the pCMV-GFP-$AR_{WT}$ using site-directed mutagenesis PCR. The reporter plasmid pARE-LUC (241B, an androgen response element driven firefly luciferase reporter vector) was constructed[31]. The pRL-TK (Renilla Luciferase Control Reporter Vectors) was purchased from Promega. COS-1 cells were cultured in Dulbecco's Modified Eagle's Medium (Gibco, Life Technologies) supplemented with 10% fetal bovine serum (Gibco, Life Technologies), 2mM L-glutamine with or without 50U/ml penicillin-streptomycin. Dihydrotestosterone (DHT) was purchased from Selleck and dissolved with dimethyl sulfoxide (DMSO) to concentrations of 1nM, 10nM and 100nM.

## Transactivation assay of eight AR mutant proteins

The transactivation activity of wildtype androgen receptor and eight novel AR variants were measured in transiently transfected COS-1 cells. $5\times10^4$ COS-1 cells/per well were seeded on 48 well plates in complete medium and cultured overnight up to 60-70% confluence. The cells were transfected with 400ng pCMV-GFP-$AR_{WT}$ or pCMV-GFP-ARmut, 400ng pARE-LUC, 4ng pRL-TK using Lipofectamine 3000 (Invitrogen) as directed by the manufacturer. After 1h, DHT with concentration of 1nM, 10nM, 100nM was added into the medium. After incubation for 48h, the cells were lysed and assayed using the Dual-Luciferase® Reporter Assay System (Promega). The ratio of Firefly to Renilla luciferase units was measured using a Turner TD-20/20 luminometer (Turner Designs, Sunnyvale, CA). Each experiment was done triplicate in at least three independent experiments, the transcription activity of wildtype AR at 100nM DHT was taken as 100% and the residual activity of eight AR variants expressed relative to that.

## Nuclear localization of the AR mutant protein K718X

$5\times10^4$ COS-1 cells/per well were seeded on 8-well glass slide (Merck-Millipore) in medium without penicillin-streptomycin and cultured overnight up to 60-70% confluence. The COS-1 cells were transfected with 500ng pCMV-GFP-empty (NC), pCMV-GFP-$AR_{WT}$ or pCMV-GFP-$AR_{K718X}$ plasmid using Lipofectamine 3000 (Invitrogen). After 24h incubation, cells were treated with 100nM DHT or DMSO. One hour after DHT stimulation, the 8-well glass slide was washed with PBS three times and fixed in 4% paraformaldehyde for 20min. After washing the slide with PBS, the nuclei were stained with Antifade Mounting Medium with DAPI (Beyotime, China) and then observed and photographed under a laser confocal microscope (Fluoview FV1000; Olympus, Japan).

## Statistical analysis

Continuous variables were presented as median (IQR) and frequency (%) for categorial variables. The transcription activity of AR wildtype and eight novel variants was performed with GraphPad Prism (version 8.0).

### Abbreviations

| | |
|---|---|
| AIS | Androgen insensitivity syndrome |
| AR | Androgen receptor |
| DSD | Disorder of sex development |
| CAIS | Complete insensitivity syndrome |
| PAIS | Partial insensitivity syndrome |
| MAIS | Mild insensitivity syndrome |
| ARE | Androgen receptor element |
| NTD | N-terminal domain |
| DBD | DNA-binding domain |
| LBD | Ligand-binding domain |
| T | Testosterone |
| LH | Luteinizing hormone |
| FSH | Follicle-stimulating hormone |
| ACMG | American College of Medical Genetics and Genomics |
| WT | Wildtype |
| DHT | Dihydrotestosterone |
| DMSO | Dimethyl sulfoxide |

## Declarations

## Ethical approval and consent to participate

The study was approved by Ruijin Hospital Ethics Committee, Shanghai Jiao Tong University School of Medicine. All patients and/or their parents signed an informed consent for the genetic study.

## Consent for publication

All authors agreed with the publication of the manuscript.

## Availability of data and materials

The supporting data in the current study are available from the corresponding author on reasonable request.

## Competing interests

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of this study.

## Funding

This study was sponsored by the grants from The National Key Research and Development Program of China (2016YFC0901503), National Science Foundation of China (81770782,81700686), Foundation of Health and Family planning Commission of Shanghai (20174Y0204), Clinical Research Plan of SHCD, State Project For Essential Drug Research and Development from Ministry of Science and Technology (2017ZX09304007002).

## Authors’ contributions

Wencui Wang: Methodology, Validation, Formal analysis, Writing-Original Draft. Sichang Zheng: Resources, Writing-Original Draft. Yu Zhao: Methodology, Validation. Zhihan Wan: Methodology. Rulai Han: Resources. Yan Qi: Resources. Guang Ning: Supervision. Shouyue Sun: Resources, Writing - Review & Editing, Supervision. Lei Ye: Methodology, Formal analysis, Writing - Review & Editing, Supervision. Weiqing Wang: Supervision.

## Acknowledgements

We are grateful to the participants for their consent to participate in this study. We would like to thank all the staff at the department of Endocrine and Metabolic Diseases who contributed to this work.

# Authors' information

Wencui Wang, Email: isunny1014@163.com

Sichang Zheng, Email: jianxianxuzhu@163.com

Yu Zhao, Email: iampetty@163.com

Zhihan Wan, Email: pamelawan@126.com

Rulai Han, Email: hanrulai@126.com

Yan Qi, Email: qyb1085@rjhn.com.cn

Guang Ning, Email: gning@sibs.ac.cn

Shouyue Sun, Email: ssy10926@rjh.com.cn

Lei Ye, Email: lei_yelei@163.com

Weiqing Wang, Email: wqingw61@163.com

# Footnotes

Wencui Wang and Sichang Zheng contributed equally to this work.

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## Figures

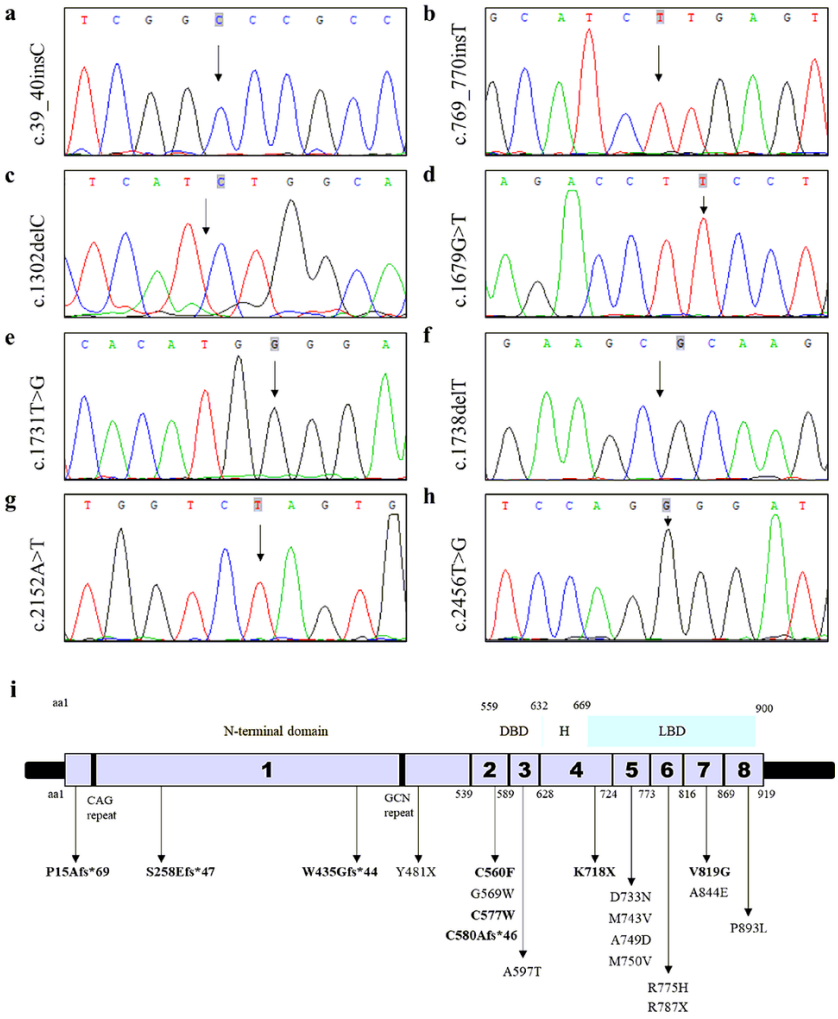


**Figure 1**

Identification of AR variants in the study. a The novel AR variant c.39_40insC (p.Pro15Alafs*69) in case 1. b The novel AR variant c.769_770insT (p.Ser258Glufs*47) in case 2. c The novel AR variant c.1302delC (p.Trp435Glyfs*44) in case 3. d The novel AR variant c.1679G>T (p.Cys560Phe) in case 5. e The novel AR variant c.1731T>G (p.Cys577Trp) in case 6. f The novel AR variant c.1738delT (p.Cys580Alafs*46) in case 7. g The novel AR variant c.2152A>T (p.Lys718Ter) in case 8. h The novel AR variant c.2456T>G (p.Val819Gly) in case 18. i Schematic representation of AR gene and the 19 AR variants. DBD, DNA-binding domain; H, hinge; LBD, ligand-binding domain. The black arrow indicated the location of the nucleotide change.

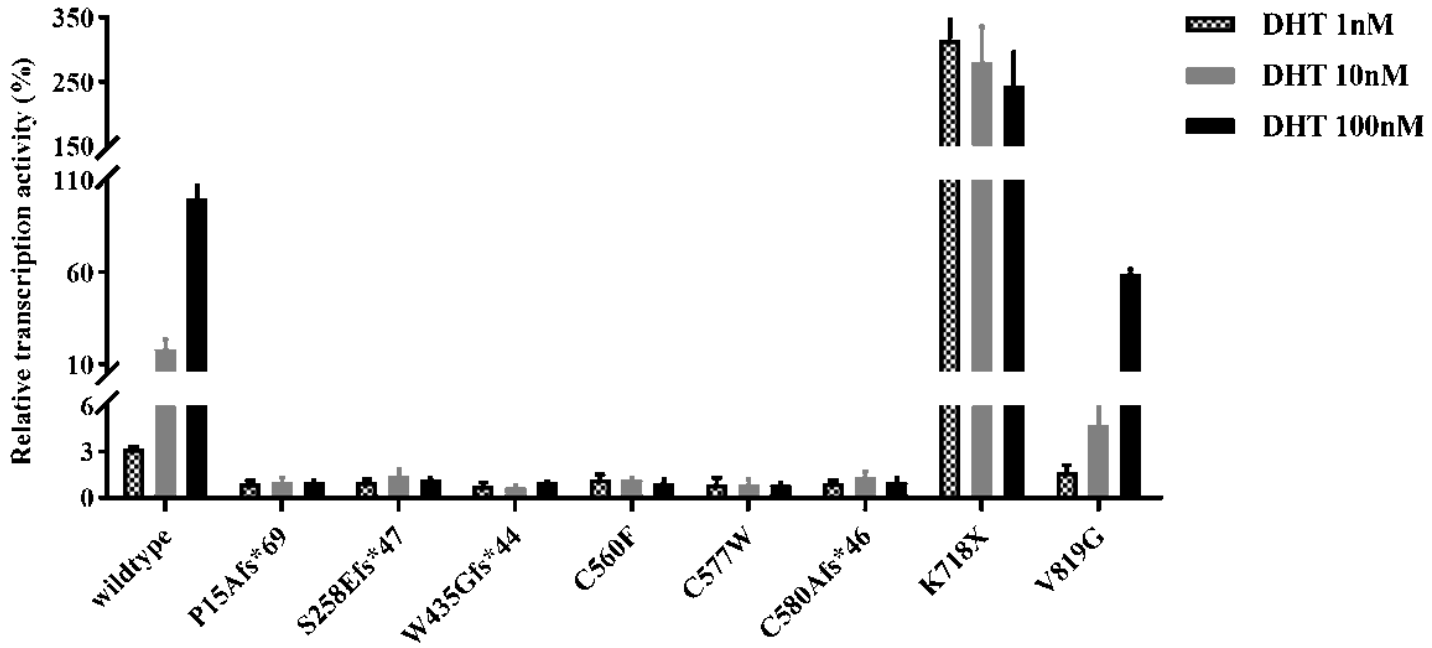


Figure 2

Transcription activity analysis of eight AR mutant proteins induced by DHT (1-100nM). Each experiment was done triplicate in at least three independent experiments, the transcription activity of wildtype AR at 100nM DHT was taken as 100%.

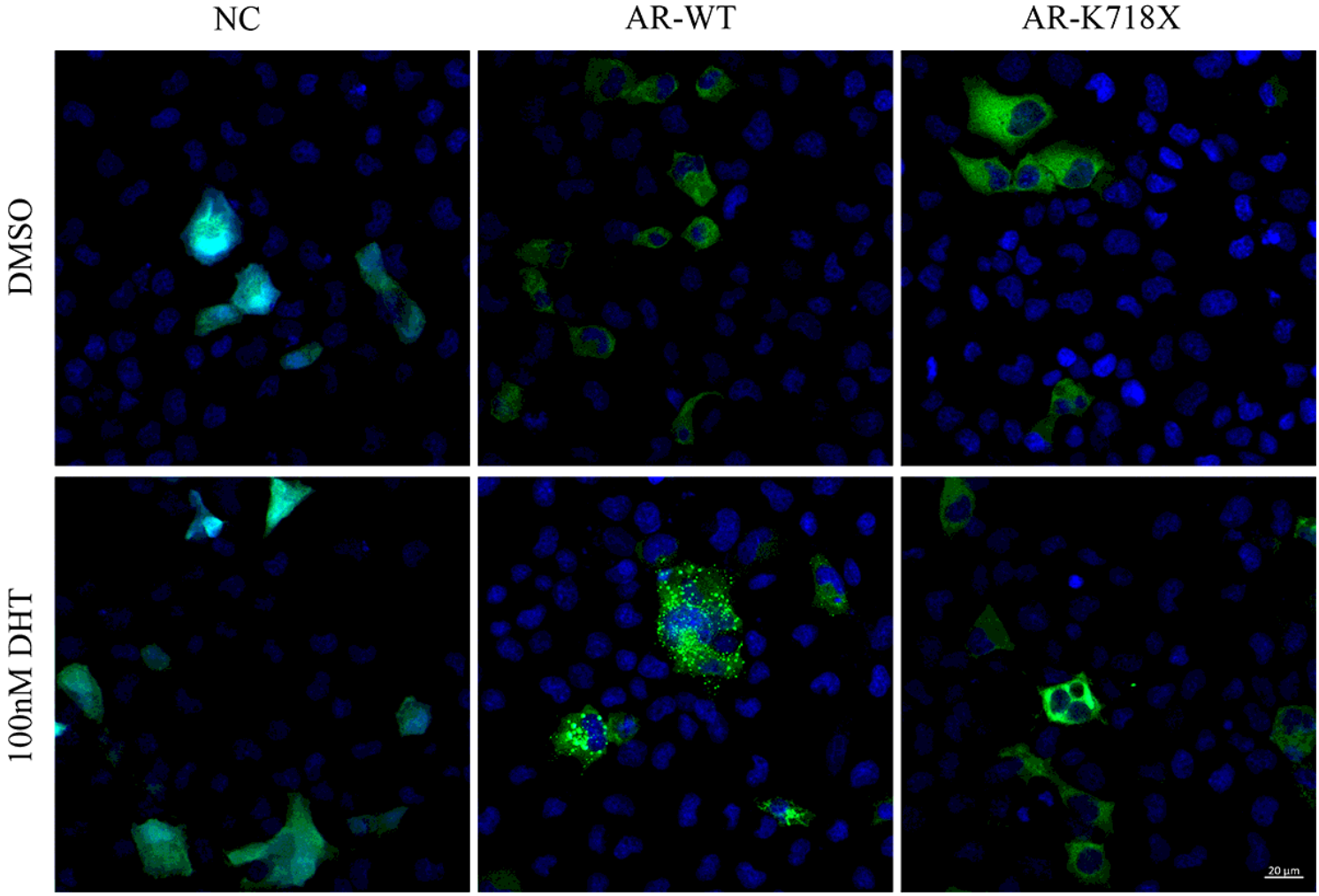

**Figure 3**

Nuclear localization of AR protein (wildtype and K718X). COS-1 cells were treated with DMSO and DHT at 100nM.