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Diverse phenotypes and fertility outcomes of patients with androgen insensitivity syndrome in a Chinese family harboring identical *AR* gene variant

Hao Geng[1,3,4†], Dongdong Tang[1,3,4†], Kuokuo Li[1,3,4], Chuan Xu[1,3,4], Chao Wang[1,3,4], Xiansheng Zhang[5*], Xiaojin He[2,4*] and Yunxia Cao[1,3,4*]

Abstract

Background Androgen insensitivity syndrome (AIS) is a rare genetic disorder characterized by resistance to androgens, mainly due to mutations in the androgen receptor (*AR*) gene. It can manifest as complete AIS, partial AIS and mild AIS. While there have been studies linking specific *AR* gene mutations to AIS phenotypes, different clinical AIS phenotypes are also reported in patients with the same *AR* gene mutation. So far, the precise correlations between phenotypes and genotypes remain incompletely understood.

Methods We conducted a thorough investigation involving four patients diagnosed with different types of AIS from a single Chinese family. Clinical manifestations, laboratory examinations, and fertility outcomes were well-documented. Furthermore, we performed genetic sequencing to detect possible pathogenetic variants.

Results Whole exome sequencing identified a hemizygous missense variant (c.2263T > C; p.Phe755Leu) of *AR* gene in all four affected patients with different degrees of undermasculinisation and heterogeneous spermatogenesis. The proband, diagnosed with partial AIS, opted for treatment with donated sperm due to non-obstructive azoospermia, while their older sibling, diagnosed with complete AIS, was raised as a girl. His two maternal uncles were both diagnosed with mild AIS, the older uncle fathered two girls naturally, whereas the younger uncle utilized assisted reproductive technology to conceive a boy because of severe oligoasthenozoospermia.

Conclusion Our study first identified the same *AR* variant (c.2263T > C;p.Phe755Leu) in four affected patients displaying highly diverse phenotypes of AIS and fertility outcomes, thereby significantly expanding the phenotypic spectrum of AIS. Notably, we presented a clear insight into different fertility outcomes of AIS patients with identical

†Hao Geng and Dongdong Tang contributed equally to this work.

*Correspondence:
Xiansheng Zhang
xiansheng-zhang@126.com
Xiaojin He
xiaojinhe@sjtu.edu.cn
Yunxia Cao
caoyunxia6@126.com

Full list of author information is available at the end of the article

AR (c.2263T > C;p.Phe755Leu) variant, which provided reliable evidence that males harboring this variant may obtain biological offspring naturally or in combination with assisted reproductive technology. Furthermore, our study underscored the potential role of androgen concentration in shaping the phenotypic diversity of AIS, warranting further investigation.

Keywords Androgen insensitivity syndrome, *AR*, Phenotype diversity, Fertility outcomes, Androgens concentration

Background

Androgen insensitivity syndrome (AIS, MIM: 300068) is one of the most common congenital disorders of sexual differentiation (DSD) in 46, XY individuals, which is mainly caused by complete or partial resistance to androgens [1]. Patients with AIS exhibit a wide phenotypic spectrum ranging from complete female external phenotype to nearly normal male external genitalia with impaired fertility. According to the phenotypic heterogeneity of external genitalia, AIS is categorized into three main types: complete AIS (CAIS, characterized by typically female external genitalia but lacking a uterus and ovaries), partial AIS (PAIS, manifested as almost female external genitalia, ambiguous external genitalia, or predominantly male external genitalia), and mild AIS (MAIS, presenting with normal male genitalia but with infertility or slight undervirilization) [2].

Androgens, including testosterone (T) and dihydrotestosterone (DHT), are essential for male sexual differentiation, development of secondary sexual characteristics, and initiation of spermatogenesis through binding to the androgen receptor (AR) [3]. Therefore, the absence or dysfunction of AR, caused by pathogenic mutations in the *AR* gene which are inherited in an X-linked recessive manner, stands as a key molecular underpinning of AIS [4]. The *AR* gene (MIM:313700), mapped on Xq11-12, consists of eight exons and encodes a protein of 920 amino acid residues featuring four major functional domains: an N-terminal domain (NTD, serving as the transcriptional activation region), a DNA-binding domain (DBD), a C-terminal ligand-binding domain (LBD, involved in binding to androgens), and a hinge region connecting LBD and DBD [5]. Upon binding to androgens, the activated AR protein complex transfers from the cytoplasm into the nucleus, subsequently stimulates transcription of downstream androgen responsive genes, and eventually elicits biological functions [6].

The pathogenic mutations within the *AR* gene commonly disrupt the normal structure of AR protein, affecting its ability to bind with ligands or downstream responsive elements, thereby leading to the occurrence of AIS [2, 7, 8]. As documented in the Human Gene Mutation Database (HGMD) (https://www.hgmd.cf.ac.uk/), approximately 600 mutations in the *AR* gene have been reported to be related to AIS, of which missense mutations occurring in LBD or DBD are the most common. Additionally, other mutations such as insertions, deletions, or splicing, are also recorded. Different types of mutations are reported to be associated with diverse phenotypes of AIS. Notably, different phenotypes of AIS with the same *AR* gene mutation have also been previously described [9–13]. Moreover, cases of fertile AIS patients (usually diagnosed with MAIS) are observed in the real world [10, 11, 14]. However, the underlying correlations between phenotypes and genotypes in AIS remain not entirely elucidated.

In this study, we observed diverse intrafamilial variabilities of AIS phenotypes in the degrees of undervirilization and spermatogenesis from four affected patients within a single Chinese family. Genetic sequencing identified the same missense hemizygous variant (NM_000044.3:c.2263T > C;p.Phe755Leu) of *AR* gene in all affected individuals.

Methods

Subjects and clinical investigations

Four patients diagnosed with AIS from a single Chinese family were enrolled in this study. The proband (III-2) attended our reproductive center for fertility consultation because of azoospermia at the age of 31 year. He had a history of unilateral cryptorchidism and had undergone orchidopexy. Upon a face to face consultation and discussion regarding family history, we established that the proband's sibling (III-1) displayed complete female phenotype with primary amenorrhea, and that two maternal uncles presented with varying degrees of undervirulisation. His younger uncle (II-5) presented almost normal secondary sexual characteristic but experienced fertility problems. Conversely, the proband's older uncle (II-3), who had a clinical phenotype similar to that of the younger uncle (II-5), fathered two girls naturally. No additional relevant conditions were detected in other family members. Clinical manifestations, physical examinations, and laboratory tests were well-recorded for all available family members. In our study, AIS was classified into CAIS, PAIS, and MAIS based on the detailed clinical characteristics. The pedigree is illustrated in Fig. 1A.

Informed consent was obtained from all participating family members, approval for conducting this study was taken from the Ethics Committee of the First Affiliated Hospital of Anhui Medical University.

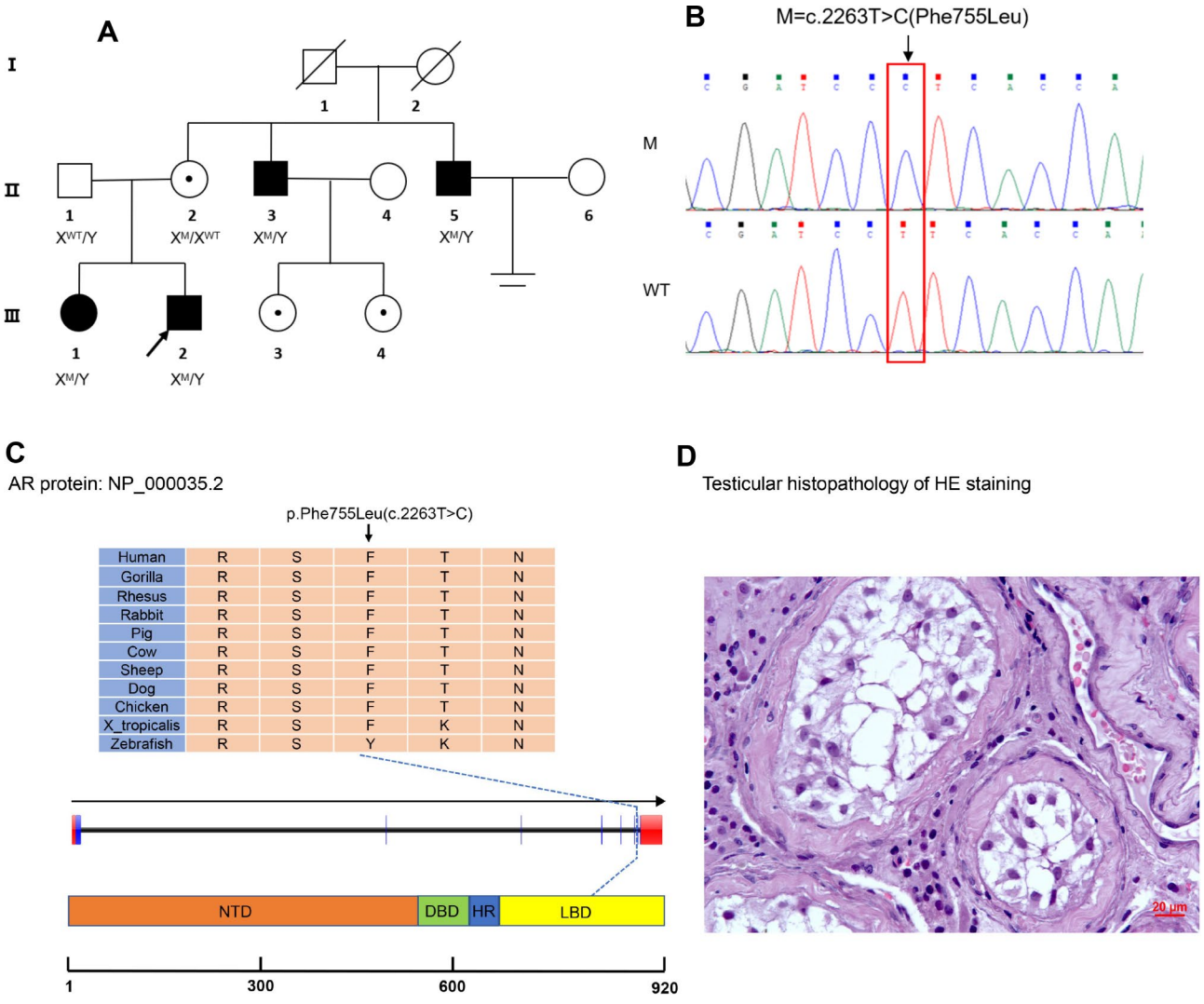

Fig. 1 Identification of *AR* variant in Chinese patients with different phenotypes of AIS. (**A**) Pedigree of the family. The proband is indicated by a black arrow. Black symbols: affected individuals with AIS; Black spots: heterozygous carriers; WT: wild type; M: mutated variant. (**B**) All the variants were verified by Sanger sequencing and the variant positions are indicated by a red box. (**C**) Variant location and conservation analysis of mutated residues in AR protein. This variant is highly conserved across different species. Color squares symbols different domains of AR protein. NTD: N-terminal domain; DBD: DNA binding domain; HR: hinge region; LBD: ligand binding domain. (**D**) Testicular histopathology of H&E staining from the proband indicated the total absence of germline cells in seminiferous tubules, conformed to the diagnosis of Sertoli cell-only syndrome

Semen analyses and sex hormone examinations

Semen samples were collected by masturbation from patients III-2 and II-5 after 2–7 days of sexual abstinence, and analyzed according to the Fifth WHO Guidelines. Serum sex hormones, including follicle stimulating hormone (FSH), luteinizing hormone (LH), oestradiol (E2), and testosterone (T) from patients II-1, II-2, II-3, II-5, III-1, and III-2, were measured with an automated Elecsys immunoanalyzer (Beckman Coulter, CA, USA) following the manufacturer's instructions.

Whole exome sequencing and bioinformatics analyses

The peripheral blood samples of the proband and his family members (II-1, II-2, II-3, II-5, and III-1) were collected to extract genomic DNA using a DNA Blood Mini Kit (Qiagen, Germany). Whole exome sequencing (WES) of the proband was performed by a commercial provider (BGI Co., Ltd., Shenzhen, China) on the Illumina HiSeq X-10 platform. Details of the method were described previously [15]. The mutation filtering criteria were listed as follows: (1) mutation frequency below 1% in the ExAc_all, 1KGP, and GnomAD databases; (2) loss-of-function mutations, including start-gain, stop-gain, frameshift, and splice site mutations; (3) high pathogenicity predicted by SIFT, PolyPhen-2, Mutation Taster, and CADD. Finally, Sanger sequencing was conducted for mutation verification in all available family members, and the primers are listed in Table S1.

Microdissection testicular sperm extraction and hematoxylin and eosin staining

After thorough consideration of treatment options and genetic counseling, the proband opted to undergo microdissection testicular sperm extraction (micro-TESE) combined with intracytoplasmic sperm injection (ICSI) for achieving fertility goal. The detailed procedures were referred to Zhang et al [16]. Fresh sperm would be used for ICSI on the day of oocyte pick-up if successful. Meanwhile, the testicular tissue sample of proband was obtained for histopathological examination by hematoxylin and eosin (H&E) staining, as described previously [17].

Results

Identification of the same hemizygous missense variant of *AR* gene in all four AIS patients

WES performance of the proband identified a hemizygous missense variant (NM_000044.3: c.2263T>C;p.Phe755Leu) in the *AR* gene (Table 1). No other pathogenic variant was found in the *AR* gene or other 46, XY DSD-related genes, including *SRY* (MIM:480000), *SOX9* (MIM:608160), *NR5A1* (MIM:184757), *WT1* (MIM:607102), *DMRT1* (MIM:602424), *MAP3K1* (MIM:600982), *CYP17A1* (MIM:609300), *SRD5A2* (MIM:607306), *ZFPM2* (MIM:603693), *CBX2* (MIM:602770), *LHCGR* (MIM:152790), and so on [18]. Additionally, WES result didn't indicate any genetic defect/alteration in known *AR* co-factors/regulators (such as *APOD*, *SRC1*, *ARA54*, *RNF4*, *TIF2* and *HDAC1*). Subsequent Sanger sequencing verified that this missense variant was inherited from his heterozygous mother, and his father was wild-type at this site. This new variant was absent in the 1KGP, ExAc_all, and GnomAD databases, and predicted to be disease-causing, possibly damaging, and damaging by Mutation Taster, polyphen-2, and SIFT, respectively. Moreover, multiple amino acid sequence alignments revealed that this residue of the AR protein was highly conserved across species, which suggested the importance of this residue in maintaining the protein function (Fig. 1B and C). Notably, this hemizygous missense variant of *AR* gene was also validated in the proband's sibling (III-1) and two maternal uncles (II-3 and II-5). Given that the proband's mother (II-2) was heterozygous for this variant, we speculated that this pathogenic variant was inherited from his departed grandmother (I-2) according to the pedigree structure (Fig. 1A).

Table 1 *AR* gene variant identified in a Chinese family with AIS

Gene	AR
DNA change	c.2263T> C/g.172945T> C
Amino acid alteration	p.Phe755Leu
Variant type	Missense
Allele Frequency	
1KGP	0
ExAc_all	0
GnomAD	0
Function prediction	
Mutation Taster	Disease causing (1.000)
Polyphen-2	Possibly damaging (0.836)
SIFT	Damaging (0.003)
CADD	Damaging (24.3)

NCBI accession number of AR is NM_000044.3; NP number of AR protein: NP_000035.2; Genome reference assembly: GRCh37/ hg19; AIS: androgen insensitivity syndrome; 1KGP: 1000 Genomes Project; ExAc_all: all the data of Exome Aggregation Consortium; GnomAD: Genome Aggregation Database

Diverse phenotypes of affected AIS patients harboring identical *AR* gene variant

Four affected patients were identified to carry the same *AR* gene variant (c.2263T>C; p.Phe755Leu). The proband (III-2) had a history of unilateral cryptorchidism, and subsequently had undergone orchidopexy. Physical examinations revealed small testes (8 ml), a micropenis (stretched length:4.5 cm; width:1.5 cm), gynaecomastia, and scant pubic and axillary hair. His older sibling (III-1) was assigned and reared as a girl, presenting a typical female phenotype with primary amenorrhea, along with bilateral inguinal hernias identified upon physical examination. His two maternal uncles (II-3 and II-5) exhibited almost normal male sexual secondary characteristics, except for micropenis (stretched length:5 cm; width:2 cm), mild gynaecomastia, and scant pubic and axillary hair. All available individuals (II-1, II-2, II-3, II-5, III-1, and III-2) underwent sex hormone tests. Basal levels of sex hormones for individuals II-1 and II-2 were within the normal range. The affected patients II-3, II-5, III-1, and III-2 showed elevated T and E_2 levels. The levels of LH in all affected patients increased or were in the upper limit of the normal range. Patients III-1 and III-2 exhibited abnormally increased FSH levels. Therefore, based on clinical manifestations, laboratory tests, and physical examinations, the proband was diagnosed with PAIS, his old sibling was diagnosed with CAIS, whereas his two maternal uncles were both diagnosed with MAIS. Further details are summarized in Table 2.

Different fertility outcomes of AIS patients harboring identical *AR* gene variant

Despite that the semen sample was not obtained, the older uncle (II-3) of proband fathered two girls naturally. While the younger uncle (II-5) was infertile because of severe oligoasthenozoospermia (SOZ), and conceived a son by utilizing assisted reproductive technology. As for the proband with non-obstructive azoospermia, no spermatozoa were retrieved from the seminiferous tubules collected from micro-TESE. H&E staining revealed the total absence of germline cells in seminiferous tubules

Table 2 Clinical characteristics and laboratory examinations of patients with AIS

Patients	Social sex	Phenotype	Clinical feathers	Ultrasound examination	Semen analyses	FSH (IU/L)	LH (IU/L)	T (nmol/L)	E_2 (pmol/L)
III-1	Female	CAIS, infertile	Primary amenorrhea, well-developed breasts, scant pubic and axillary hair, small labial folds, a short blind-ending vagina, inguinal hernia	Inguinal testes, no uterus and ovaries	N/A	18.91	7.79	31.36	349
III-2	Male	PAIS, infertile	Unilateral cryptorchidism, micropenis, small testes, scant pubic and axillary hair, gynaecomastia	N/A	Azoospermia	21.54	8.00	32.27	338
II-3	Male	MAIS, fertile	Micropenis, scant pubic and axillary hair, mild gynecomastia	N/A	N/A	5.43	9.01	51.97	570
II-5	Male	MAIS, infertile	Micropenis, scant pubic and axillary hair, mild gynecomastia	N/A	SOZ	8.12	10.59	55.48	497

FSH: Follicle-stimulating hormone =1.4-18.1 IU/L; LH: Luteinizing hormone= 1.5-9.3 IU/L; T: Testosterone=8.36-28.77nmol/L; E_2= Estradiol=0-191pmol/L; N/A= not available; SOZ: severe oligoasthenozoospermia; AIS: androgen insensitive syndrome

(Fig. 1D), confirming the diagnosis of Sertoli cell-only syndrome [19]. Finally, the proband received treatment with donated sperm.

Discussion

It is reported that approximately 600 different mutations distributed throughout the whole *AR* coding sequence are responsible for the occurrence of AIS in the HGMD database. Various mutation types have been linked to the clinical diversity observed in AIS phenotypes. Complete loss-of-function mutations occurring in the NTD or LBD severely disrupt the AR protein function, often leading to the occurrence of CAIS. Partial disruptions in *AR* activity, mainly caused by missense mutations in the LBD or DBD, typically present as PAIS. While the mutations leading to MAIS are predominantly missense and located in NTD, which results in the reduced transactivational activity of the AR protein [2, 9]. Notably, diverse AIS phenotypes with identical *AR* gene mutation have also been reported previously. For example, Liu et al. reported the same missense mutant of LBD (p.Tyr764His) in patients with CAIS and PAIS [12]. Holterhus et al. identified the same p.Leu712Phe mutant in four patients with varying degrees of defective virilization [13]. Moreover, Chu et al. identified the same p.Arg840Cys substitution of AR protein in a large Chinese AIS family, resulting in highly intrafamilial variable phenotypes in external genitalia virilization and spermatogenesis [10]. However, the correlations between the genotypes and AIS phenotypes remain still unclear.

In the present study, we identified the same *AR* gene variant (c.2263T>C; p.Phe755Leu) in four AIS patients from a single Chinese family with highly diverse clinical phenotypes and fertility outcomes, which expanded the phenotypic spectrum of AIS. The four affected patients displayed highly variable intrafamilial phenotypes in the degree of undervirilization. Concerning fertility issues, individuals with CAIS are typically assigned and reared as girls, with a consistent female gender identity [20–22]. In this study, the sibling of proband (III-1), diagnosed with CAIS, was raised as a girl and prepared to undergo orchiectomy. As reported previously, most PAIS patients were infertile and had to select donated sperm or opt for adoption [1, 23, 24]. In the pedigree reported here, the PAIS-affected proband exhibited non-obstructive azoospermia, with no sperm detected in the seminiferous tubules collected from micro-TESE, necessitating the use of donated sperm. In addition, several previous studies demonstrated that slightly diminished size of penis was the most severe clinical manifestation among fertile AIS patients [11, 25–27]. The older uncle (II-3) who displayed a micropenis and scant body hair, was fertile in our study. However, the younger uncle (II-5), presenting slightly similar undervirilization to Patient II-3, was infertile due to impaired sperm production (SOZ), but eventually conceived a boy with the use of ICSI technology. Moreover, in the study conducted by Chu et al. in a large family with AIS, one affected patient with hypospadias and gynecomastia exhibited normal semen density and motility, whereas another affected patient with azoospermia exhibited only slight undervirilization [10]. This phenomenon suggested that the impaired fertility potential of AIS patients is not consistent with the degree of external genitalia undervirilization. Furthermore, it is possible for some AIS cases to conceive naturally or in combination with assisted reproductive technology [28, 29]. However, it should be noted that genetic counseling is essential in this context, considering the risk of transmission of the *AR* gene mutation. Besides, sex hormone tests showed that the LH levels in four affected patients increased or were in the upper limit of the normal range, thus reflecting impaired androgen actions. The fertile older uncle (II-3) had normal levels of FSH and LH, consistent with several previous studies [10, 11, 25–27]. However, the infertile patients with azoospermia (III-2) and SOZ (II-5) showed abnormally elevated sex hormone levels and

impaired spermatogenesis. Further studies should focus on the correlations between the sex hormone levels and fertility potential in AIS patients.

The nonsynonymous p.Phe755Leu (c.2265 C>A) mutant of AR protein was first reported in PAIS by Hiort et al. in 1994 [30]. Structural and functional studies conducted by Tadokoro et al. suggested that Phe755 of AR was highly conserved among mammalian species, and that p.Phe755Leu mutant exhibited decreased binding capacity to androgens as well as transcriptional activity in vitro [7]. Additionally, Lobaccaro et al. identified the p.Phe755Val mutant in patients with CAIS, and no androgen binding capacity was detected in genital skin fibroblasts, indicating a complete absence of the *AR* function [31]. Furthermore, the identical p.Phe755Ser mutant in PAIS and MAIS patients was also reported, indicating the partially impaired *AR* function [29, 32]. These studies indicated that the phenotypic heterogeneity can be partially attributed to the different types of mutation occurring at codon 755, resulting in various degrees of reduction in androgen binding capacity and/or transcriptional activation.

Several studies have reported the association between the p.Phe755Leu (c.2265 C>A) mutant and PAIS (as detailed in Table S2) [30, 32, 33]. However, in our study, although the nucleotide alteration differed (c.2263T>C), we identified the same amino acid change (p.Phe755Leu) in four affected patients with diverse phenotypes (CAIS, PAIS, and MAIS) from a single Chinese family. Thus, phenotypic heterogeneity of AIS with identical *AR* variant indicated that other factors, independent of *AR* sequence alterations, contributed to the diverse AIS phenotypes. Recently, several factors have been reported to account for the variable phenotypes observed in AIS cases sharing identical *AR* mutations. Firstly, differences in individual 5α-reductase activities may lead to distinct external genitalia phenotypes [34, 35]. Secondly, the post-translational modifications of AR protein, such as phosphorylation, acetylation, and methylation, were proven to influence AR activity [36, 37]. Thirdly, numerous co-regulatory factors, including both positive (coactivators) and negative (corepressors) regulators, interact with AR, thereby affecting its function [38]. Moreover, the concentration-related androgen action has been proven critical for normal male sexual development and spermatogenesis. Significant alterations have been observed in the binding capacity or transcriptional activity of AR mutant with different concentrations of androgen in vitro [7, 12, 13, 39]. Additionally, in the real world, a good clinical response to high-dose androgen therapy in genitalia masculinization has been observed in some AIS cases [13, 28, 40–42]. Therefore, different concentrations of the ligand are regarded as another mechanism that may play a significant role in the individual phenotypic diversity of AIS.

Notably, in this study, we identified the same p.Phe755Leu mutant of *AR* in four affected AIS patients. The severely affected CAIS and PAIS patients showed mildly increased serum concentrations of T, whereas the two MAIS patients with slight undermasculinization displayed higher serum T concentrations. Tadokoro et al. reported that the p.Phe755Leu mutant led to a reduction in the androgen binding affinity and a severe impairment of the transactivation activity at low DHT concentration, whereas the defective transactivation activity could be compensated in the presence of high concentration of DHT [7]. Our results further supported that the concentration of androgens may be another important factor compensating for impaired AR function. Further insights should be gained into the specific role of androgens concentration involved in the phenotypic diversity of AIS with the p.Phe755Leu mutant.

There are several limitations to consider in this study. Firstly, we failed to obtain genital skin fibroblasts to detect the androgen binding capacity of mutated AR protein in any affected patients. Secondly, we did not conduct functional analysis of *AR* p.Phe755Leu mutant in vitro, as described previously, to further confirm the significant changes in *AR* activity at different androgen concentrations. Additionally, as all affected patients refused to receive a high-dose androgen therapy, we are unable to inform the clinical response to endocrine therapy in *AR* p.Phe755Leu mutant cases. Finally, the specific mechanism by which different androgen concentrations affect AR protein activity with the p.Phe755Leu mutation remains unclear. These limitations underscore the need for further research to address these unresolved questions and enhance our understanding of AIS pathogenesis and treatment.

Conclusion

Our study first identified the same *AR* variant (c.2263T>C;p.Phe755Leu) in four affected patients with highly diverse phenotypes of AIS and fertility outcomes, which significantly expanded the phenotypic spectrum of AIS with this substitution. Androgens concentration may play an important role in the phenotypic diversity of AIS, and deserve further investigation. Furthermore, we presented a clear insight into different fertility potential of AIS patients with the *AR* p.Phe755Leu, which provided the evidence that males harboring this variant may obtain biological offspring naturally or in combination with assisted reproductive technology. Notably, it should be cautiously accompanied by genetic counseling considering the risk of transmission of the *AR* gene mutation.

Abbreviations

AIS	Androgen insensitivity syndrome
AR	androgen receptor
DSD	Disorders of sexual differentiation

NTD	N-terminal domain
DBD	DNA-binding domain
LBD	ligand-binding domain
FSH	Follicle stimulating hormone
LH	Luteinizing hormone
E2	Oestradiol
T	Testosterone
DHT	Dihydrotestosterone
WES	Whole exome sequencing
Micro-TESE	Microdissection testicular sperm extraction
ICSI	Intracytoplasmic sperm injection
SOZ	Severe oligoasthenozoospermia

Supplementary Information

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Supplementary Material 1

Supplementary Material 2

Acknowledgements
Not applicable.

Author contributions
Hao. Geng., Xiaojin. He., Xiansheng. Zhang., and Yunxia. Cao. designed the study. Hao. Geng., Chuan. Xu. and Chao.Wang. collected the samples and data. Hao. Geng., Dongdong.Tang. and Chuan. Xu. performed the experiments. Dongdong. Tang. and Kuokuo. Li. analyzed the data. Hao. Geng. and Dongdong. Tang. wrote the manuscript. Xiaojin. He., Xiansheng. Zhang. and Yunxia. Cao revised the draft. All authors read and approved the final manuscript.

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Data availability
The datasets utilized in this study can be obtained on reasonable request from the authors.

Declarations

Ethics approval and consent to participate
All participants approved and signed written informed consents upon enrolment. And this study was approved by the Ethics Committee of the First Affiliated Hospital of Anhui Medical University(P2020-12-36) and conducted in accordance to the Declaration of Helsinki.

Consent for publication
Not applicable.

Competing interests
The authors declare no competing interests.

Author details
[1]Reproductive Medicine Center, Department of Obstetrics and Gynecology, The First Affiliated Hospital of Anhui Medical University, No 218 Jixi Road, Hefei, Anhui 230022, China
[2]Reproductive Medicine Center, Department of Obstetrics and Gynecology, Shanghai General Hospital, Shanghai Jiao Tong University School of Medicine, Shanghai 200000, China
[3]Anhui Province Key Laboratory of Reproductive Health and Genetics, No 81 Meishan Road, Hefei, Anhui 230032, China
[4]NHC Key Laboratory of Study on Abnormal Gametes and Reproductive Tract, Anhui Medical University, No 81 Meishan Road, Hefei, Anhui 230032, China
[5]Department of Urology, The First Affiliated Hospital of Anhui Medical University, No 218 Jixi Road, Hefei 230022, China

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