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ORIGINAL ARTICLE

Male Endocrinology

Phenotypic and molecular characteristics of androgen insensitivity syndrome patients

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Androgen insensitivity syndrome (AIS), an X-linked recessive genetic disorder of sex development, is caused by mutations in the androgen receptor (*AR*) gene, and is characterized by partial or complete inability of specific tissues to respond to androgens in individuals with the 46,XY karyotype. This study aimed to investigate *AR* gene mutations and to characterize genotype–phenotype correlations. Ten patients from unrelated families, aged 2–31 years, were recruited in the study. Based on karyotype, altered hormone profile, and clinical manifestations, nine patients were preliminarily diagnosed with complete AIS and one with partial AIS. Genetic analysis of *AR* gene revealed the existence of 10 different mutations, of which five were novel (c.2112 C>G[p.S704R], c.2290T>A[p.Y764N], c.2626C>T[p.Q876X], c.933dupC[p.K313Qfs*28], and c.1067delC[p.A356Efs*123]); the other five were previously reported (c.1789G>A[p.A597T], c.2566C>T[p.R856C], c.2668G>A[p.V890M], c.2679C>T[p.P893L], and c.1605C>G[p.Y535X]). Regarding the distribution of these mutations, 60.0% were clustered in the ligand-binding domain of *AR* gene. Exons 1 and 8 of *AR* gene each accounted for 30.0% (3/10) of all mutations. Most of the truncation mutations were in exon 1 and missense mutations were mainly located in exons 4–8. Our study expands the spectrum of *AR* gene mutations and confirms the usefulness of *AR* gene sequencing to support a diagnosis of AIS and to enable prenatal or antenatal screening.

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Keywords: androgen insensitivity syndrome; androgen receptor; disorder of sex development; mutation

INTRODUCTION

Androgen insensitivity syndrome (AIS; OMIM# 300068) is a common 46,XY disorder of sex development (DSD) resulting from complete or partial resistance to the biological actions of androgens. Affected individuals typically exhibit inguinal swelling during infancy or primary amenorrhea during puberty.[1] According to the degree of androgen responsiveness, AIS presents with a broad spectrum of defects in the external genitalia and can be subdivided into three phenotypes: complete androgen insensitivity syndrome (CAIS) with typical female external genitalia, partial androgen insensitivity syndrome (PAIS) with predominantly male or ambiguous external genitalia, and mild androgen insensitivity syndrome (MAIS) with typical male external genitalia or an isolated micropenis, but with gynecomastia at puberty and infertility in adulthood.[2] Of these categories, CAIS is the classic manifestation of AIS. The clinical diagnosis of CAIS is typically based on primary amenorrhea at puberty or inguinal hernia and labial swelling in a female infant with a 46,XY karyotype.[1] A pathogenic mutation of the androgen receptor gene (*AR*; OMIM# 313700) is the only established molecular cause of the X-linked recessive inherited disease.

The *AR* gene is located on chromosome Xq11-12 and contains eight exons that encode a protein of 920 amino acid residues. This protein functions as a steroid hormone-activated transcription factor and contains four major functional domains: the N-terminal domain (NTD, transcriptional activation region encoded by exon 1), the DNA-binding domain (DBD, encoded by exon 2 and 3), and finally, the ligand-binding domain (LBD, encoded by exon 4–8), which is involved in binding to androgens and relevant co-activator proteins. Upon binding of the hormone ligand, the androgen receptor protein dissociates from accessory proteins, translocates into the nucleus, dimerizes, and subsequently stimulates the transcription of androgen-responsive genes.[3,4] Thus, normal primary male sexual development before birth and the development of secondary sexual characteristics during puberty require the presence of a functional androgen receptor.

To date, more than 1000 different AIS-causing variants in *AR* gene have been identified.[5,6] Among these, missense mutations are the most common which primarily occur in the AR-DBD or AR-LBD, leading to impairment in DNA or androgen binding, respectively. Splicing, small insertions and deletions, and nonsense mutations have also been reported, which result in a premature stop codon.[5]

In this study, we report the clinical characteristics and the molecular genetic analysis of *AR* gene in ten unrelated patients suffering from AIS. Sequence analysis of the AR gene identified the disease-associated mutations. Our study expands the spectrum of mutations associated with AIS and may contribute to treatment and reproductive counseling for these patients.

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PATIENTS AND METHODS

Ten unrelated patients from ten nonconsanguineous families attending the Reproductive and Genetic Hospital of Citic-Xiangya (Changsha, China) during 2013–2016 were included in the study. Based on the karyotype, altered hormone profile, and clinical manifestations such as external genitalia, primary amenorrhea, inguinal hernia, and scant or absent pubic and/or axillary hair, nine patients were preliminarily diagnosed with CAIS. One case was suspected of having PAIS because of the ambiguous external genitalia. This study was approved by the institutional ethics committee of the Reproductive and Genetic Hospital of Citic-Xiangya, and written informed consent was obtained from all adult patients or the parents of pediatric patients.

Mutation analysis of the AR gene

Genomic DNA was extracted from the peripheral blood lymphocytes of patients using the QIAamp DNA blood Midi kit (QIAGEN, Hilden, Germany). All the eight exons and the intron–exon boundaries of *AR* gene were amplified by PCR using specific primers designed with Primer 5 software (**Table 1**). The 50-μl PCR reaction mixture included 25 μl of GoTaq Green Master Mix (2×, Promega, Madison, WI, USA), 22 μl of RNase-free water, 50 ng of DNA, and 10 μmol l^{-1} of each primer. Amplification reactions were performed in a thermocycler set at 95°C for 5 min (initial denaturation) followed by 35 cycles of denaturation at 94°C for 40 s, annealing at the appropriate temperatures for 40 s, and extension at 72°C for 40 s, with a final extension at 72°C for 10 min. The amplified PCR products were analyzed using 2.0% agarose gel electrophoresis to determine the band size. Subsequently, bi-directional sequencing of the amplified PCR products was performed using an ABI 3730 automated sequencer (Applied Biosystems, Forster City, CA, USA).

Table 1: Primers used for androgen receptor gene amplification and sequencing

Primer name	*Exon*	*Sequence (5′—3′)*	*Annealing temperature (°C)*	*Product size (bp)*
AR-e1A	1	F: CGACTACCGCATCATCACAG	60	432
		R: CTCATCCAGGACCAGGTAGC		
AR-e1B	1	F: CACAGGCTACCTGGTCCTGG	60	416
		R: CTGCCTTACACAACTCCTTGGC		
AR-e1C	1	F: CTTAAGCAGCTGCTCCGCT	59	596
		R: CCAGAGCCAGTGGAAAGTTG		
AR-e1D	1	F: TAGACGACAGCGCAGGCAAG	60	963
		R: AGAATCTGGGAGGGCAGGAAT		
AR-e1E	1	F: TGGAGAACCCGCTGGACTAC	59	525
		R: CCGCTAGATACCCCAGAACAC		
AR-e2	2	F: CAGTGACATGTGTTGCATTG	58	229
		R: AGAAAGGGAAAGAGAAGTGC		
AR-e3	3	F: TGGAAACTCATTATCAGGTCTA	58	204
		R: GAGAGAGGAAGGAGGAGGAA		
AR-e4	4	F: GTTTAGAGTCTGTGACCAGGGAG	63	506
		R: GGCAGAAAAGCACCAGACAT		
AR-e5	5	F: GGATGCCCGAATACCAGAG	63	339
		R: CTAAGCTTCACTGTCACCCCA		
AR-e6	6	F: GGCAATCAGAGACATTCCCT	63	303
		R: GCTGGCTTTTCCCTAATAATG		
AR-e7	7	F: ACAGGAAGCCAAGTAGATGGT	60	336
		R: CTCTATCAGGCTGTTCTCCCT		
AR-e8	8	F: GAAACAAAAGGCTGAAAGAC	60	374
		R: CATCAATAGAGGAAATTCCCC		

AR: androgen receptor; e: exon; F: forward; R: reverse

Nucleotide sequences were analyzed using the DNASTAR software package (Madison, WI, USA), and the results were compared with reference sequences GenBank NG_009014.2 (*AR*, g.DNA), GenBank NM_000044.2 (*AR*, c.DNA), and GenBank NP_000035.2 (*AR*, p.protein) through BLAST searches. The human gene mutation database, the NCBI dbSNP database (http://www.ncbi.nlm.nih.gov/SNP), and the 1000 Genomes Project (http://www.internationalgenome.org/1000-genomes-browsers/) were used to determine whether the detected mutations had been reported previously.

Prediction of AR mutation effects

The potential pathogenicity of the novel *AR* mutations was examined by *in silico* analysis using three different software packages: Polyphen-2 (http://genetics.bwh.harvard.edu/pph2) and SIFT (http://sift.bii.a-star.edu.sg/) for missense mutations and Mutation Taster (http://www.mutationtaster.org/) for all mutations.

RESULTS

Clinical features

The clinical features and hormone data of the ten patients are presented in **Table 2**. Among these patients, six (aged 16–31 years) visited their physician owing to primary amenorrhea, three (aged 2–18 years) for inguinal hernia, and the remaining one (aged 9 years) for severe hypospadias; all had a 46,XY karyotype. All patients presented with typical female external genitalia and were diagnosed with suspected CAIS, with the exception of Case 3 who presented severe hypospadias and a presumptive diagnosis of PAIS.

For the postpubertal patients, other major clinical manifestations were as follows: well-developed breasts, scant or absent pubic and/or axillary hair, absent/rudimentary Müllerian structures (including uteri and ovaries), and inguinal/abdominal testes. All patients underwent hormone testing, except Case 3. The levels of most of the serum hormones, including follicle-stimulating hormone (FSH), luteinizing hormone, estradiol, and testosterone, were within or elevated above the normal male reference range, as shown in **Table 2**. Cases 5, 6, and 8 underwent gonadectomy, and histological analysis of the gonadal tissues of Case 8 showed prepubertal tubules and large areas of fibrosis within the gonads. No germ cells were observed in either gonad (**Figure 1**). The data of Cases 5 and 6 were not available. Case 3 had been raised as a boy and was reported previously.[7] He was preliminarily clinically diagnosed with PAIS because of ambiguous external genitalia, including perineal hypospadias, unilateral cryptorchidism, and phallocampsis. Cases 1, 5, 7, and 8 had one or more relatives with a similar phenotype, whereas the others were sporadic. Family pedigrees of Cases 1, 5, 7, and 8 based on Standardized Human Pedigree Nomenclature[8] are shown in **Figure 2**.

Molecular genetic analysis of the AR gene

Sequence analysis revealed that all ten patients harbored *AR* mutations. Ten different mutations, including six missense mutations, two nonsense mutations, one duplication, and one deletion, were found. The number of CAG and GGC repeat in exon 1 of *AR* gene ranged from 20 to 26 and 13 to 17, respectively (**Table 3**). The two novel missense mutations were: c.2112C>G (p.S704R), identified in Case 1, and c.2290T>A (p.Y764N), detected in Case 2. The remaining four known missense mutations, c.1789G>A (p.A597T), c.2566C>T (p.R856C), c.2668G>A (p.V890M), and c.2679C>T (p.P893L), which led to the change of a single amino acid, were identified in Cases 3, 4, 5, and 6, respectively. A known nonsense mutation (c.1605C>G (p.Y535X)) in exon 1 was detected in Case 7. Case 8 had a novel C>T substitution at site c.2626 that changed the glutamine (CAG) at 876 to a stop codon (TAG) (p.Q876X). In the family of Case 8, the mother and a

Table 2: Clinical characteristics and hormone levels of the ten patients with androgen insensitivity syndrome

Case	Age (year)	Phenotype	Karyotypes	Reason for consultation	Main clinical features			Ultrasound examination		FSH (mIU ml^{-1})	LH (mIU ml^{-1})	E2 (pg ml^{-1})	T (ng ml^{-1})
					Development of breasts	Pubic-axillary hair	Uterus	Gonads					
1	19	Female	46,XY	Primary amenorrhea	Yes	Absent	None	Inguinal testes		14.45	35.61	62.65	23.44
2	26	Female	46,XY	Primary amenorrhea	Yes	Scant/absent	None	Inguinal testes		18.52	44.30	28.99	5.86
3	9	Male	46,XY	Hypospadias	/	/	None	Unilateral inguinal testes and other side normal		n.d.	n.d.	n.d.	n.d.
4	2	Female	46,XY	Inguinal hernia	/	/	None	Inguinal testes		31.43	1.81	<10.00	<0.45
5	18	Female	46,XY	Inguinal hernia	Yes	Scant/absent	None	Inguinal testes		26.08	25.19	34.35	11.53
6	26	Female	46,XY	Primary amenorrhea	Yes	Scant	None	Inguinal testes		n.d.	n.d.	n.d.	3.50
7	16	Female	46,XY	Primary amenorrhea	Yes	Absent	Rudimentary uterus	Abdominal testes		3.61	17.97	48.00	8.19
8	31	Female	46,XY	Primary amenorrhea	Yes	Absent	Rudimentary uterus*	Abdominal testes		2.53	13.94	34.71	4.39
9	22	Female	46,XY	Primary amenorrhea	Yes	Absent	Rudimentary uterus	Rudimentary ovary*		8.10	27.70	78.15	4.03
10	2.5	Female	46,XY	Inguinal hernia	/	/	None	Inguinal testes		10.95	4.82	24.35	8.93

FSH reference range: 1.5–12.4 mIU ml^{-1}; LH reference range: 1.7–8.6 mIU ml^{-1}; T reference range: 2.49–8.39 ng ml^{-1}; E reference range: 7.63–42.6 pg ml^{-1}; /: no data due to prepuberty; n.d.: no data. *Suspected. FSH: follicle-stimulating hormone; LH: luteinizing hormone; T: testosterone; E: estradiol

Figure 1: Histological analysis of the gonadal tissues of Case 8, obtained by gonadectomy. Large areas of prepubertal tubules (arrow) surrounded by some fibrosis (arrowhead) are visualized, with no germ cells in the gonads. Scale bar = 25 μm.

maternal aunt of the proband were heterozygous for this mutation. A maternal cousin and another maternal aunt who presented with similar clinical features also had the same mutation (**Table 3**). Case 9 presented with a duplication mutation, c.933dupC, which changed lysine to glutamine at codon 313 and introduced a premature stop at codon 341 (c.933dupC (p.K313Qfs*28)). Case 10 harbored the single nucleotide deletion c.1067delC, leading to a frameshift and a truncated protein containing only 479 amino acids (c.1067delC (p.A356Efs*123)). The two frameshift mutations were in exon 1 and are reported for the first time in this study. Familial analysis showed that the affected relatives of Case 1, 5, 7, and 8 also harbored the same *AR* mutation of the proband. The detailed results for the patients and their available family members are shown in **Figures 2**, **3** and **Table 3**.

Prediction of the effects of the novel mutations

All detected novel mutations were evaluated as "disease causing" by Mutation Taster. The amino acid changes c.2112C>G (p.S704R) and c.2290T>A (p.Y764N) were predicted as "probably damaging" with a score of 1.000 (sensitivity: 0.00 and specificity: 1.00) by PolyPhen-2 and "not tolerated" with scores of 0.01 and 0.02, respectively (<0.05 suggests potential pathogenicity), by Sorting Intolerant From Tolerant (SIFT) analysis.

DISCUSSION

AIS is commonly found in individuals with 46,XY DSD, and CAIS is the classic manifestation of the disease. In this study, we investigated the genetic causes of ten patients with AIS and identified ten different mutations of *AR* gene, including six missense and four truncation mutations, which led to a premature stop codon and were predicted to result in the synthesis of truncated proteins. Sixty percentage of the identified mutations were detected in LBD, and exons 1 and 8 of the *AR* gene each accounted for 30.0% (3/10) of all mutations (**Table 3** and **Figure 4**). Among these ten detected mutations, p.S704R, p.Y764N, p.Q876X, p.K313Qfs*28, and p.A356Efs*123 have not been described previously in the literature.

Table 3: Summary of androgen receptor gene mutations in the ten Chinese patients with complete androgen insensitivity syndrome

Case	*Base change*	*AA change*	*Location*	*Type of mutation*	*Previous study*	*Heterozygote carriers*	*Other members affected*	*CAG*	*GGC*	*Prediction of novel mutation effects*		
										Mutation taster	*PolyPhen-2*	*SIFT*
1	c.2112 C>G	S704R	Exon 4	Missense	No	Mother	One sister	20	16	Disease causing	1.000	0.01
2	c.2290T>A	Y764N	Exon 5	Missense	No	n.d.	None	21	16	Disease causing	1.000	0.02
3	c.1789G>A	A597T	Exon 3	Missense	Yes	Mother	None	20	16	-	-	-
4	c.2566C>T	R856C	Exon 7	Missense	Yes	Mother, grand mother	None	22	16	-	-	-
5	c.2668G>A	V890M	Exon 8	Missense	Yes	Mother, 1 maternal aunt	One sister, 1 maternal cousin	22	13	-	-	-
6	c.2679C>T	P893L	Exon 8	Missense	Yes	n.d.	None	25	16	-	-	-
7	c.1605C>G	Y535X	Exon 1	Nonsense	Yes	Mother	One maternal aunt	26	16	-	-	-
8	c.2626C>T	Q876X	Exon 8	Nonsense	No	Mother, 1 maternal aunt	One maternal cousin, 1 maternal aunt	20	17	Disease causing	-	-
9	c.933dupC	K313Qfs*28	Exon 1	Insertion	No	n.d.	None	21	16	Disease causing	-	-
10	c.1067delC	A356Efs*123	Exon 1	Deletion	No	n.d.	None	20	16	Disease causing	-	-

PolyPhen-2: sensitivity, 0.00; specificity, 1.00. SIFT: Sorting Intolerant From Tolerant. <0.05 suggests potential pathogenicity. n.d.: no data; -: No data due to known mutation or frameshift mutation; AA: amino acid

Figure 2: Partial pedigrees of the families 1, 5, 7, and 8. The proband is indicated by an arrow. Black symbols: affected individuals; open symbols: unaffected individuals; black spots: carriers; squares: males; circles: females.

Exon 1 of the *AR* gene contains two polymorphic trinucleotide repeats CAG and GGC, which encode polyglutamine and polyglycine tracts, respectively. These polyamino acids play an important role in the N-terminal transactivation domain of the AR protein. In a healthy population, the number of CAG and GGC repeats varies from 8 to 33 and 10 to 27, respectively.[9,10] Some studies have shown a relationship between the two polymorphic microsatellite regions of the receptor and its transactivational activity.[9,11] In this study, the number of CAG repeats ranged from 20 to 26, whereas the number of GGC repeats ranged from 13 to 17; the most frequent number of repeats was 20 and 16, respectively (**Table 3**). Therefore, it appears that there is no correlation between the function of these mutant proteins and the number of both repeats in these patients.

The p.S704R mutation, resulting from a C>G transition in exon 4 (c.2112 C>G), led to a change from serine (AGC) to arginine (AGG) at codon 704 and was predicted to be pathogenic by *in silico* mutation analysis software. Although this mutation has not been reported previously, similar missense mutations at the same codon have been described elsewhere. The mutations p.S704C (c.2110A>T) and p.S704I (c.2111G>T) have been identified in patients with CAIS.[12,13] Moreover, another single nucleotide change, p.S704G (c.2110A>G), was found in a patient with PAIS with ambiguous external genitalia.[14] Thus, the codon at residue 704 seems to be a critical position for AR protein functionality.

The other novel missense mutation identified in this study, c.2290T>A(p.Y764N), is located in exon 5 of *AR* gene and was detected in Case 2. Other mutations at codon 764 have also been found previously. Mutation p.Y764H caused by a T>C substitution at site c. 2290 was found to be causative of CAIS,[15] while the recurrent mutation p.Y764C was first described in a PAIS family by McPhaul *et al*.[16] and has since been frequently reported in Brazil[17,18] and the United Kingdom.[19] The nonsense mutation p.Y764X was also detected in the same codon of a CAIS individual.[20] These studies indicate that the codon at residue 764 might be a mutational hot spot. The hinge region and LBD of AR are essential for dimerization of the AR protein.[21,22] Mutations in these regions could impair the function of the receptor by disrupting dimer formation and impairing AR-induced transactivation.[23,24] Thus, the two novel missense mutations in the present study may interfere with receptor dimerization, resulting in CAIS.

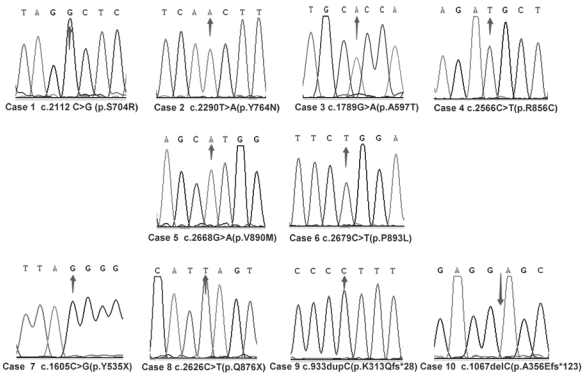

Figure 3: Sanger sequence traces of the ten mutations are depicted. The position of the *AR* gene mutation is indicated by an arrow. *AR*: androgen receptor.

The c.1789G>A(p.A597T) mutation in the *AR* gene was detected in Case 3, who was the only PAIS patient in the study and suffered from hypospadias, penis deformity, and cryptorchidism. Gast *et al*.[25] previously demonstrated that this single amino acid exchange could result in PAIS as an effect of DNA binding of the androgen receptor. This appears to suggest that a small modification of the androgen receptor binding has a defined phenotype. However, phenotypes of PAIS are highly variable and clinically indistinguishable from other 46,XY DSD diseases, such as 5α-reductase type 2 deficiency caused by *SRD5A2* mutations and 17β-hydroxysteroid dehydrogenase type 3 deficiency resulting from a *HSD17B3* defect. In our study, mutations in *SRD5A2* gene and *HSD17B3* gene-coding regions of Case 3 were not detected (data not shown).

The other three previously described missense mutations were identified in AR-LBD, which is important for binding to androgen and relevant co-activator proteins. The p.R856C mutation was found in Case 4 and confirmed to affect the binding of the androgen receptor and ligand.[26,27] In Case 5, the p.V890M mutation, which was described as p. V889M in an earlier study, occurred at a CpG hot spot and could lead to reduced androgen binding.[28] Moreover, the mutant receptor has a higher ligand dissociation rate and is unstable.[29] Another known mutation in Case 6, p.P893L, has repeatedly been reported as a cause of CAIS.[30–32]

Two nonsense mutations were also found in the present study: the previously reported mutation p.Y535X in Case 7 and the novel mutation p.Q876X in Case 8. These mutations led to a premature stop codon in exons 1 and 8, generating truncated proteins containing only 535 and 876 amino acids, respectively. *In vitro* functional assays in a previous study by McPhaul *et al*. revealed that the p.Y535X mutation decreased the androgen binding to approximately zero.[33] In our study, p.Q876X was the only truncation mutation detected in the LBD of the AR protein, in which nonsense mutations are rare.[5] Both the two nonsense mutations led to truncated AR proteins, which were expected to affect androgen binding and could explain the cause of AIS in these families.

Two novel frameshift mutations were detected in the study, including a duplication mutation and a deletion mutation. Duplication mutations of *AR* gene are rare in AIS patients,[5] but Case 9 was shown to harbor a c.933dupC mutation. The deletion mutation, c.1067delC, was detected in Case 10. Both frameshift mutations were located in exon 1 and were predicted to cause truncated AR proteins that lacked their essential functional domains (DBD or LBD).

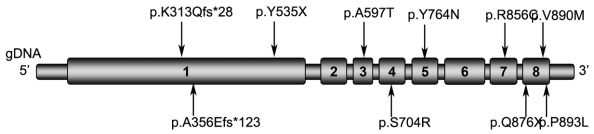

Figure 4: Schematic diagram showing the *AR* gene and the localization of the ten mutations identified in the present study. The exons of the *AR* gene are indicated by numbered boxes. *AR*: androgen receptor.

In the present study, most of the detected missense mutations were located in the LBD and led to CAIS. The possible reason may be that the major missense mutations occurring in NTD or DBD, which usually lead to MAIS or PAIS,[5] had a mild effect on AR function so that these patients were easily missed or misdiagnosed with other 46,XY DSD diseases. In addition, missense mutations in the LBD mainly cause CAIS because of disruptions of the AR protein function such as interference with ligand binding, disruption in dimer formation, or impaired AR-induced transactivation.[23] In the present study, however, most truncation mutations, including p.Y535X, p.K313Qfs *28, and p.A356Efs*123, were identified in the NTD. Compared with single amino acid changes, truncation mutations in the NTD, including nonsense and frameshift mutations, are considered to be more intolerable. This is because of the loss of many crucial functional domains and the disruption of *AR* gene expression through the introduction of nonsense codons, which induce nonsense-mediated mRNA decay.[34,35] Although no functional studies have been performed for these truncation mutations, mutant proteins lacking several key regions could be pathogenic or degraded, or confer a loss of function.

CONCLUSION

Our molecular diagnosis identified ten different pathogenic *AR* gene mutations in ten patients with AIS. Our study expands the spectrum of *AR* gene mutations and could provide evidence for the genetic and reproductive counseling of families with AIS.

AUTHOR CONTRIBUTIONS

SMY conducted the genetic studies, drafted the initial manuscript, and wrote the manuscript; YNZ participated in data collection and sequence alignment, performed the initial analyses, and approved the final manuscript as submitted; JD and WL conceived of the study; CFT and LLM participated in data collection; GL and GXL critically reviewed the manuscript; YQT conceived of the study, helped draft the initial manuscript, and wrote the manuscript. All authors read and approved the final manuscript.

COMPETING INTERESTS

All authors declare no competing interests.

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