Original Article

Detection of Molecular Variations at Androgen Receptor Gene in 46,XY Differences in Sex Development Cases

Nanis S. Marzuki, Hannie D. Kartapradja, Farah N. Coutrier, Irfan Wahyudi[1], Jose R.L. Batubara[2]

Eijkman Research Center for Molecular Biology, Research Organization for Health, National Research and Innovations Agency, Cibinong, Departements of [1]Urology and [2]Child Health, Faculty of Medicine, Cipto Mangunkusumo Hospital - Universitas Indonesia, Jakarta, Indonesia

Abstract

Introduction: One of the common causes of 46,XY differences in sex development (DSD) cases is androgen insensitivity syndrome. This X-linked recessive inherited condition is associated with pathological variations of the AR gene, leading to defects in androgen action. Affected 46,XY infants or individuals experience variable degrees of undervirilization and those with severe form will have female-like external genitalia. Therefore, they were more likely assigned and reared as females. The confirmatory molecular test is often needed due to similar clinical manifestations with other conditions causing 46,XY DSD. Since in our country, the molecular test for the AR gene is lacking, the study is conducted as a preliminary study to elaborate on the possibility of developing a molecular test for the AR gene in 46,XY DSD cases. **Methods:** Archived DNAs of 13 46,XY DSD cases were analyzed using polymerase chain reaction and direct sequencing for molecular defects in the AR gene. Clinical and hormonal data were collected and analyzed. **Results:** The study successfully amplified and visualized the eight exons of the AR gene and revealed two subjects carrying AR gene variants at exon 7. In the first case, 1.2-year-old boy carried heterozygous p.Gln825Arg, which has never been reported elsewhere, and the second subject, a 2.1-year-old girl with heterozygous p.Arg841His. Both subjects presented with severe undervirilization of external genitalia with external genitalia masculinization scores (EMS) of 1.5 and 3. **Conclusion:** In this series, two of 13 46,XY DSD cases carried variants at the AR gene, resulting in complete androgen insensitivity syndrome.

Keywords: 46,XY DSD, androgen insensitivity syndrome, AR gene

Introduction

Androgens play essential roles in the development and differentiation of sex during fetal life and postnatal. In expressing its biological action, androgens need intact functional androgen receptors, which are nuclear receptors encoded by the AR gene. Defects in the AR gene may lead to androgen insensitivity syndrome (AIS), which clinically presents with undervirilization in the affected 46,XY infants/individuals. This rare condition is included in the spectrum of 46,XY differences in sex development (DSD). The clinical manifestations vary from micropenis, hypospadias, undescended testis, and ambiguous genitalia to female-appearance external genitalia. The severe form of AIS or complete AIS (CAIS) presents with predominantly female external genitalia and therefore affected 46,XY newborn infants may be assigned as girls at birth. These girls may develop breasts at a pubertal age. However, they fail to have menstruation and may not have children due to the absence of the uterus. The milder form of AIS is partial AIS (PAIS), which manifests clinically with ambiguous genitalia, hypospadias, undescended testis, micropenis, or infertility.[1,2] This condition rarely occurs, with a prevalence estimation of 1:20,000 to 1:99,000 births, and is inherited in an X-linked recessive manner.[2,3] Our experience running a cytogenetic laboratory found that nearly half of the cases referred for cytogenetic analysis were DSD cases and 51.2% were 46,XY DSD. Several reports described around 24% to 65% of the 46,XY DSD cases were caused by AIS.[3-6]

Access this article online

Quick Response Code:

Website: https://journals.lww.com/indjem/

DOI: 10.4103/ijem.ijem_257_23

Address for correspondence: Dr. Nanis S Marzuki, Gedung Widyasatwaloka, Cibinong Science Center, Jl. Raya Jakarta Bogor Km 46, Cibinong, Kabupaten Bogor, West Java, Indonesia.
E-mail: andi008@brin.go.id; sacharina99@gmail.com

Submitted: 15-Jun-2023 **Revised:** 31-Jul-2023
Accepted: 24-Sep-2023 **Published:** 29-Apr-2024

This is an open access journal, and articles are distributed under the terms of the Creative Commons Attribution-NonCommercial-ShareAlike 4.0 License, which allows others to remix, tweak, and build upon the work non-commercially, as long as appropriate credit is given and the new creations are licensed under the identical terms.

For reprints contact: WKHLRPMedknow_reprints@wolterskluwer.com

How to cite this article: Marzuki NS, Kartapradja HD, Coutrier FN, Wahyudi I, Batubara JR. Detection of molecular variations at androgen receptor gene in 46,XY differences in sex development cases. Indian J Endocr Metab 2024;28:197-200.

The AR gene is located at the Xq11-12 chromosome, comprises eight exons over 90 kb in length, and encodes a 920 amino acid protein. The gene consists of three functional domains, which include the N terminal domain, which is located at exon 1 and acts as a modulator; the DNA binding domain, which lies at exon 2 and 3 and expands 152 and 117 bp long; and ligand binding domain (LBD), which is located at exon 5 to exon 8 and has about 151-288 bp long.[2,3]

Our 12 years (2010-2021) of experience in the molecular test for DSD cases revealed that among 2,964 cases referred for karyotyping, 17.27% were chromosomal DSD, whereas 1,201 cases (40.5%) were 46,XY DSD. Further molecular study on 170 46,XY DSD cases detected 63 cases that carried deleterious SRD5A2 mutations, 17 cases with SRY gene defects, 11 with AR gene defects, and one with SOX9 mutations.

Clinicians often face difficulties in diagnosing AIS due to overlapping clinical appearances with other 46,XY DSD conditions, such as 5-alpha reductase deficiency, 17 beta-hydroxylase deficiency, congenital adrenal hyperplasia, or gonadal dysgenesis. Therefore, a molecular test to detect pathological variations in the AR gene is warranted to confirm the diagnosis. In our country, this molecular test is not yet available. Therefore, this study aimed to know the possibility of developing a method for detecting mutations in the AR gene in our setting.

Materials and Methods

The study included archived DNAs of DSD subjects with 46,XY karyotypes and having a variable degree of undervirilization, including micropenis, hypospadias, scrotum bifidum, or undescended testis. These DNAs were already screened and revealed to have a normal sequence of the SRD5A2 gene. The external genitalia appearances were graded using the EMS described by Ahmed and Rodie.[7] Subjects whose karyotype revealed other cell lines were excluded. The recorded data on age at diagnosis, EMS, and testosterone level were analyzed.

Molecular analysis of AR gene

Ten pairs of suitable forward and reverse primers, as previously defined by Nie M *et al*.[8] and Rosa S *et al*.,[9] were used to amplify the eight exons of the AR gene and its flanking regions. The PCR solution was prepared by mixing homogeneously into a polymerase chain reaction (PCR) tube and adding 75 µL of 1x MyTaqTM HS Red Mix (Bioline, USA), 0.4 nM of forward and reverse primers, and 300 ng of the DNA sample. Amplification was carried out in a PCR machine (Veriti 96-Well Thermal Cycler, Applied Biosystems, USA) with conditions suitable for the examined primer pairs. The PCR conditions for exon 1C, 1D, and 1E were performed in initial denaturation 95°C for 5 minutes, denaturation 95°C for 30 seconds, annealing 62°C for 40 seconds, elongation 72°C for 40 seconds, and final elongation 72°C for 5 minutes. The denaturation, annealing, and elongation were conducted for 35 cycles. The PCR condition was the same for other exons, while the annealing was 59°C for exons 2, 3, 4, 6, 7, and 8, and 69°C for exon 5. Thereafter, the PCR products were separated electrophoretically on 1.5% agarose gel which was prepared by dissolving agarose in 1x TBE buffer solution which was heated using a microwave. After dissolving, 5 mcL FloroSafe DNA Stain (1st Base, Singapore) was added and the gel was run for 20-30 minutes. The results of the separation of the DNA amplification were then documented using SmartDoc 2.0 (Accuris instruments, USA).

Subsequently, the PCR products were sequenced using the 96-capillary system, an automatic genetic analyzer (Applied Biosystems, USA). Furthermore, the resulting sequences were analyzed using CLC Sequence Viewer 8.0 (Qiagen) software with published reference (NCBI reference sequence: NM_000044.2).

Ethical Aspect

Participants' or parental written consent was obtained before the study started. This study protocol was approved by the Ethics Committee of the Faculty of Medicine, University of Indonesia–Cipto Mangunkusumo Hospital (approval number: KET/265/UN2.F1/ETIK/PPM.00.02/2022) on November 21, 2022.

Results

There were 13 subjects, aged 4.8 months to 24 years, included in the study. Seven subjects were reared as females, three were adults, and one was in the pubertal stage. The molecular analysis of the subjects' DNAs succeeded in amplifying and visualizing the whole coding region (exon 1-8) and its flanking intronic areas of the AR gene. Our study revealed two variants, which were both located at exon 7 of the AR gene. The clinical features and AR gene mutations carried by the two subjects are shown in Table 1.

The first variation of the AR gene, the p.Gln825Arg, was detected in a boy aged 1 year and 2 months with undermasculinized external genitalia. He had penoscrotal hypospadias, micropenis, scrotum bifidum, and unilateral undescended testis. He had prominently female phenotype external genitalia. However, the presence of the right testis led the clinician to assign the infant as male. The second case carried the second amino acid change, the p.Arg841His. She presented with severe undervirilized external genitalia, resembling female genitalia, so the infant was assigned as female at birth. The electropherograms of both mutations were demonstrated in Figures 1 and 2.

Since the p.Gln825Arg was a novel missense variant, we analyzed it further to predict the effect of this variant on the protein level. For that purpose, the variant was assessed as 'probably damaging' [Figure 3] by the Polyphen-2 software (Polymorphism Phenotyping v2, http://genetics.bwh.harvard.edu/pph2/) and predicted to 'affect protein function' (intolerant) with the score of 0.05 with SIFT software (https://sift.bii.a-star.edu.sg/).

Table 1: Clinical Features and Mutations at the AR Gene Detected in the Cases

Cases	Age (Year)	Sex of Rearing	EMS	Androgens		Mutations at the AR Gene
				T (ng/mL)	DHT (pg/mL)	
1	1.2	Male	1.5	0.10	33	p.Gln825Arg
2	2.1	Female	3.0	0.56	0.27	p.Arg841His
3	0.7	Female	0	0.13	2.00	None
4	19.0	Female	3.0	4.89	760.00	None
5	1.2	Male	3.0	0.24	44.00	None
6	3.0	Male	10.5	0.01	0.24	None
7	20.0	Female	0	7.32	968.00	None
8	13.0	Female	3.0	1.76	103.46	None
9	5.0	Male	3.0	0.20	77.54	None
10	11.4	Female	3.0	0.70	70.49	None
11	0.4	Male	3.0	0.34	5.01	None
12	24.0	Female	0	0.81	407.41	None
13	5.0	Male	3.0	1.74	189.34	None

EMS=External genitalia masculinization score; T=testosterone; DHT=dihydrotestosterone

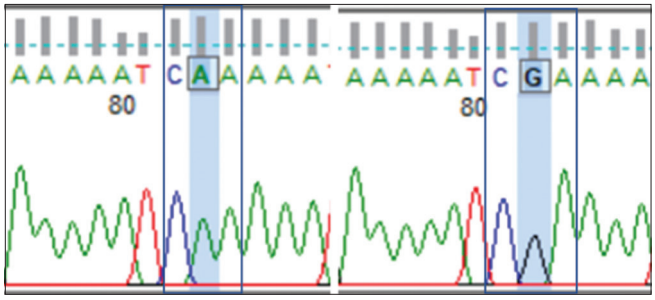

Figure 1: The electropherogram of normal sequence at codon 825 AR gene, CAA (Gln, Glutamine) (left) and its variant, CGA (Arg, Arginine) (right)

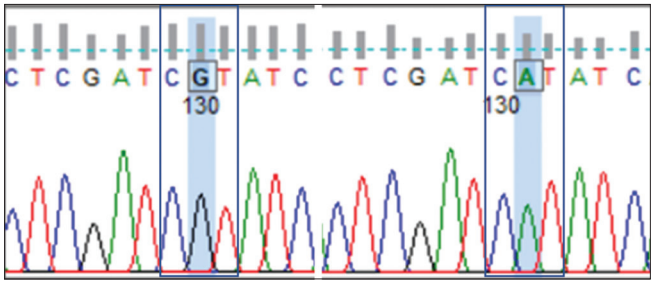

Figure 2: The electropherogram of normal sequence at codon 841 AR gene, CGT (Arg, Arginine) (left) and its variant, CAT (His, Histidine) (right)

Discussion

More than 1,100 pathogenic variations in the AR gene have been recorded in a database for the AR gene with mostly missense variants and more than 600 variants were associated with AIS.[2,3,10] Among 13 subjects included in this study, we found two subjects (15.4%) with an abnormal sequence in their AR gene. Zhu H *et al*.[6] detected 36 of 150 46,XY DSD cases (24%) carried the AR gene variants, mostly (24/26) located at LBD. Both mutations detected in our study were located at exon 7, which was known to be part of the receptor's LBD. Mutations at this site of the protein may lead to impaired binding of AR to LBD. The genotype-phenotype correlation in the AR gene variations was not obvious; some areas of the AR gene are prone to variations between 688-712, 739-784, and 827-870 amino acid residues.[2,10] One of our cases bared mutations at this site (p.Arg841His) and showed CAIS phenotypes. As described previously, the clinical manifestations of variants at exon 6-8 of the AR gene may present as PAIS or CAIS, depending on their effects on the protein function.[1,2,10] This may also be associated with the type of amino acid substitutions: glutamine to arginine and arginine to histidine. Both substitutions involved arginine, which is included in hydrophilic, large volume, polar, and positively charged amino acid classes, while the glutamine belongs to the hydrophilic, medium volume, polar, uncharged group, and the histidine is a neutral, medium volume, polar, and positively charged amino acid. These differences presumably lead to significant changes in the protein structure and, therefore, cause reduced protein activities.

The p.Gln825Arg transversion has not been reported in other populations. In-silico analysis for this variant using Polyphen-2 and SIFT software revealed that the protein was probably damaged and affected by the amino acid change. Looking at its location, it was adjacent to the area where mutations commonly occur and may present clinically as PAIS or CAIS,[2,10] as in our case presented CAIS.

Elevated levels of LH and T with gynecomastia may lead to the diagnosis of AIS in pubertal age.[1,2] However, in infancy and childhood, the LH and T levels may yield inconclusive results, as in our cases, the hormonal levels did not give additional information. As such, an hCG stimulation test should be performed. Unfortunately, the hCG preparation was lacking in our area, and therefore, during prepubertal age, the molecular test should be performed to reach the final diagnosis. Providing the precise etiology in 46,XY DSD with AIS has a significant role in sex assignment and genetic counseling, especially for female carriers.

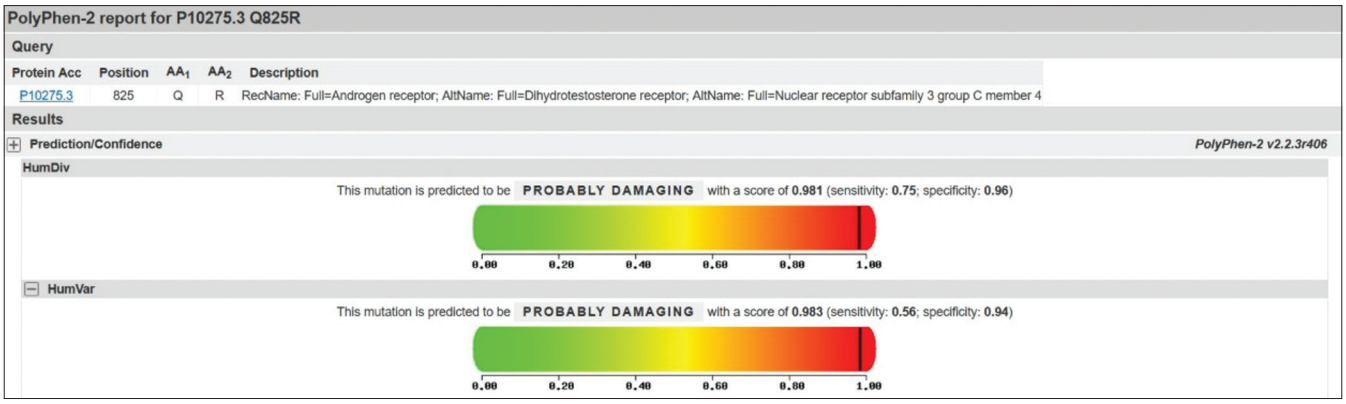

Figure 3: In-silico analysis using Polyphen-2 software on p.Gln825Arg revealed that this variant was predicted to be 'probably damaging'

Our cases carrying mutations at the AR gene showed severe undervirilized external genitalia with EMS <4, mimicking female genitalia. Despite the phenotype similarity, both cases were assigned and reared differently. Although the first case presented with EMS of 1.5, he was raised as a boy since the clinician found a testis in the left 'labia'. Four other prepubertal cases were also reared as boys, although their EMS <4. A report of 291 infants with genital ambiguity revealed a median (10^{th}-90^{th} centile) EMS of 3.5 (2-8) and 2 (1-6) for those raised as males and females, respectively.[11] To be raised as boys is uncommon in CAIS cases. Most affected 46,XY individuals with CAIS are assigned and reared as females. They mainly present late in postpubertal age due to lack of menstruation. Despite this complaint, they are convenient and maintain their gender identity as females.[1,6] Therefore, both our cases need further follow-up to assess their gender preference in the future, especially after pubertal age.

Limitations

The study had a limited sample size. We did not perform the hCG stimulation test in the prepubertal subjects.

Conclusion

The study described the development of a method to detect variation at the AR gene and found two of 13 cases of 46,XY DSD carried heterozygous variants at the AR gene, which were the p.Gln825Arg and the p.Arg841His. Further molecular analysis on the remaining 46,XY DSD cases is warranted to elaborate the final etiology.

Acknowledgment

The authors thank Firman Pratama Idris for his technical and analytical support in troubleshooting problems with DNA analysis techniques.

Authors' contribution

NSM and HDK designed and structured the study. NSM, IW, and JRLB reviewed and analyzed the clinical data. HDK and FNC performed the molecular analysis, while HDK and NSM analyzed the results. NSM and HDK drafted the manuscript, and all authors reviewed and contributed to the final manuscript.

Financial support and sponsorship

Our institution financially supported the study.

Conflicts of interest

There are no conflicts of interest.

References

1. Batista RL, Costa EMF, Rodrigues AS, Gomes NL, Faria JA Jr, Nishi MY, *et al*. Androgen insensitivity syndrome: A review. Arch Endocrinol Metab 2018;62:227-35.
2. Gulía C, Baldassarra S, Zangari A, Briganti V, Gigli S, Gaffi M, *et al*. Androgen insensitivity syndrome. Eur Rev Med Pharmacol Sci 2018;22:3873-87.
3. Boehmer ALM. Genotype versus phenotype in families with androgen insensitivity syndrome. J Clin Endocrinol Metab 2001;86:4151-60.
4. Touzon MS, Garrido NP, Marino R, Ramirez P, Costanzo M, Guercio G, *et al*. Androgen insensitivity syndrome: Clinical phenotype and molecular analysis in a single tertiary center cohort. J Clin Res Pediatr Endocrinol 2019;11:24-33.
5. Kumar A, Sharma R, Faruq M, Kumar M, Sharma S, Werner R, *et al*. Erratum: Clinical, biochemical, and molecular characterization of Indian children with clinically suspected androgen insensitivity syndrome. Sex Dev 2022;16:34-45.
6. Zhu H, Yao H, Xu Y, Chen Y, Han B, Wang N, *et al*. Phenotypic and biochemical characteristics and molecular basis in 36 Chinese patients with androgen receptor variants. Orphanet J Rare Dis. 2021;16:122.
7. Ahmed SF, Rodie M. Investigation and initial management of ambiguous genitalia. Best Pract Res Clin Endocrinol Metab 2010;24:197-218.
8. Nie M, Zhou Q, Mao J, Lu S, Wu X. Five novel mutations of SRD5A2 found in eight Chinese patients with 46,XY disorders of sex development. Mol Hum Reprod 2011;17:57-62.
9. Rosa S, Biason-Lauber A, Mongan NP, Navratil F, Schoenle EJ. Complete androgen insensitivity syndrome caused by a novel mutation in the ligand-binding domain of the androgen receptor: Functional characterization. J Clin Endocrinol Metab 2002;87:4378-82.
10. Gottlieb B, Beitel LK, Nadarajah A, Paliouras M, Trifiro M. The androgen receptor gene mutations database: 2012 update. Hum Mutat 2012;33:887-94.
11. Ahmed SF, Khwaja O, Hughes IA. The role of a clinical score in the assessment of ambiguous genitalia. BJU Int 2000;85:120-4.