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ORIGINAL RESEARCH

Preimplantation Genetic Diagnosis of Androgen Resistance Syndrome Caused by Mutation on the *AR* Gene in Vietnam

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Background: Androgen resistance syndrome or androgen insensitivity syndrome (AIS – Androgen Insensitivity Syndrome, OMIM 300068) is an X-linked recessive genetic syndrome causing disorders of sexual development in males. This disease is caused by mutations in the AR gene located on the X chromosome, which encodes the protein that structures the androgen receptor, with the role of receiving androgens. Mutation of the AR gene causes complete or partial loss of androgen receptor function, thereby androgen not being obtained and exerting its effect on target organs, resulting in abnormalities of the male reproductive system due to this organ system, differentiating towards feminization under the influence of estrogen. Disease prevention can be achieved by using pre-implantation genetic diagnosis, which enables couples carrying the mutation to have healthy offspring.

Aim: To carry out preimplantation genetic diagnosis of androgen resistance syndrome.

Methods: Sanger sequencing was used to detect the mutation in the blood samples of the couple, their son, and 01 embryo that were biopsied on the fifth day based on the findings of next-generation sequencing (NGS) of the affected son. We combined Sanger sequencing and linkage analysis using short tandem repeats (STR) to provide diagnostic results.

Results: We performed preimplantation genetic diagnosis for AIS on an embryo from a couple who had previously had an affected son. Consequently, one healthy embryo was diagnosed without the variant NM_000044: c.796del (p.Asp266IlefsTer30).

Conclusion: We report on a novel variant (NM_000044: c.796del (p.Asp266IlefsTer30)) in the AR gene discovered in Vietnam. The developed protocol was helpful for the preimplantation genetic diagnosis process to help families with the monogenic disease of AIS but wish to have healthy children.

Keywords: androgen insensitivity syndrome, AIS, AR gene, preimplantation genetic diagnosis, short tandem repeats, STR

Introduction

Androgen resistance syndrome or androgen insensitivity syndrome (AIS; OMIM 300068) is an X-linked recessive genetic syndrome causing disorders of sexual development in males. The disease is caused by mutations in the *AR* gene located on the X chromosome, which encodes for the protein forming the androgen receptor and is responsible for taking up testosterone in cells.

The *AR* gene, which consists of 8 exons and a coding sequence size of 2757 nucleotides (919 codons), is located at Xq11-q12 on the long arm of the X chromosome[1,2] There is a CAG repeat sequence in the first exon of *AR* gene. The range of CAG repeats in healthy is 11–31; however, the increased number of CAG repeats is frequently inactivated to lower androgen receptor production and results in under-masculinized males.[3] Furthermore, more than 800 variants in the AR gene have been reported to be responsible for AIS. AIS is inherited following the X-linked recessive principle; however, about 30% of AIS cases are caused by de novo mutations.[4,5]

Mutations in the *AR* gene result in a total or partial loss of androgen receptor function; then, androgen cannot affect target organs and cause abnormalities in the male reproductive system because these organs differentiate towards feminization when

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exposed to estrogen. AIS patients have male biological sex (karyotype: 46, XY), but their phenotype varies according to how much their external genitalia have become feminized including types: Complete androgen insensitivity syndrome (CAIS) is characterized by female external genitalia, absence of Mullerian structures, short vagina, abnormal testicles, and under-development of secondary sex characteristics; partially androgen insensitivity syndrome (PAIS) stands out by a sexually ambiguous phenotype, undescended testicles, poor development of secondary sex characteristics, and infertility; Mild androgen insensitivity syndrome (MAIS) has milder symptoms with under masculinization with or without preserved fertility in otherwise normal males.[5–7] The worldwide incidence of AIS is estimated to range from 1 in 20,000 to 64,000 male newborns with the CAIS type and an unknown incidence with the PAIS type.[8]

Diagnosis of AIS is based on clinical characteristics, ultrasound combined with biochemical indicators, and karyotype determined to be 46, XY to distinguish between other developmental gender disorders. Furthermore, the molecular and genetic tests searching for mutations in the *AR* gene may be used to diagnose AIS.

Having children with AIS patients is currently impossible, so treatments focus on enhancing the quality of life and preventing complications. Determining *AR* gene mutations contributes to supporting the diagnosis and prognosis of AIS patients, helping guide sex decision for patients, and genetic counseling for women carrying the mutant. In addition to early disease detection to improve quality of life and prevent possible complications, disease prevention can be achieved by using pre-implantation genetic diagnosis, which enables couples carrying the mutation to have healthy offspring. In this article, we report on the discovery of a novel variant in the *AR* gene and the implementation of pre-implantation genetic diagnosis of AIS by our research team in Vietnam.

Subjects and Methods

Subjects

Peripheral blood samples from a mother, father, and child serve as the research subjects. The child born in 2019 has a female phenotype and female external genitalia. After karyotyping revealed 46, XY, the patient was diagnosed with AIS. Next-generation sequencing – NGS was conducted on this son's peripheral blood sample, and 01 variant: *AR*:c.796del was detected. With the desire to have a healthy child without disease genes, the couple received genetic counseling and then underwent in vitro fertilization. One embryo (A1) of the couple was cultured till the fifth day and biopsied to prepare for pre-implantation genetic diagnosis.

Methods

DNA Extraction from Blood

Peripheral venous blood samples were extracted DNA using the G-spin™ Total DNA Extraction Kit (the extraction procedure was performed according to the company's instructions). Measure the purity and DNA concentration of samples using the SpectraMax QuickDrop machine. The obtained DNA was diluted with deionized water to reach a concentration of about 20 ng/μL, ensuring A260/280 purity from 1.8 to 2.0, and stored at −20°C.

Blastocyst Embryo Biopsy and Whole Genome Amplification for Embryos' Genome

A couple that underwent in vitro fertilization (IVF) had an embryo (A1) cultured till the fifth day. In order to perform an embryonic biopsy, five to ten cells from the TE layer – a section of the embryo that would eventually develop into placental tissue – had to herniate out through the opening made during zona pellucida breaching. The embryo biopsy procedure for PGT was performed according to the recommendations of the ESHRE PGT Consortium and SIG-Embryology Biopsy Working Group.[9] The embryo sample was then washed by adding PBS 1X and 1% PVP solution. After that, the washed embryo cells were contained in the 0.2 mL PCR tube and stored at −20°C. Embryo biopsy samples were DNA extracted and amplified using the REPLI-g® Single Cell Kit. Concentration and purity are guaranteed to qualify the amplified DNA of the embryo for use. DNA samples were stored at −20°C.

Embryo's Sex Determination

The primer amplifies a sequence on the *SRY* gene (Sex Determining Region of the Y Chromosome), designed to determine the sex of the embryos. The *SRY* gene encodes the SRY protein, which is involved in the transcription process in men's testicles.

Gender determination results are for research and diagnostic purposes only. The primer sequence to amplify the *SRY* gene segment with a size of 470bp designed by the research team is as follows: SRY-f: 5'-GAATATTCCCGCTCTCCGG-3'; SRY-r: 5'-GCTGGTGCTCCATTCTTGA-3'.

Polymerase Chain Reaction (PCR) Analysis

After NGS sequencing and determining the AR:c.796del variant that the son carried, we designed primers to amplify the gene segment containing the variant on exon 1 of the *AR* gene using Primer-BLAST software. The melting temperature of the primer pair is 62.9°C. The primer sequence to amplify the *AR* gene segment containing the variant with the size of 276bp is as follows: AR1-f: 5'-GCACTTCGACCATTTCTGACAAC-3'; AR1-r: 5'-CCTTTGGTGTAACCTCCCTTGAA-3'.

A total volume of 25μL for each PCR reaction with the components are as follows: 12.5μL of GoTaq Green Mastermix 2X, 5.5μL Deion water, 1.0μL of each forward and reverse primer, and 5.0μL of template DNA. Based on the melting temperature of the primer pairs, the gradient-PCR reaction is performed with the primer annealing temperature range from 50°C to 60°C, and the optimal primer annealing temperature is selected as 58°C. Thermal cycling for amplifying the segment spanning the mutation is as follows: 98°C - 2 minutes, 30 cycles (95°C - 30 seconds, 58°C - 30 seconds, 72°C - 20 seconds) and 72°C - 10 minutes. After running the PCR reaction, electrophoresis on a 3% agarose gel checked the amplified products before conducting Sanger sequencing.

Sanger Sequencing

The *AR*:c.796del variant was identified by Sanger sequencing on the SeqStudio Genetic Analyzer using the BigDye® Terminator v3.1 Cycle Sequencing kit (Thermo Fisher Scientific). Bioedit Sequence Alignment Editor was used to analyze the sequence of the amplified segment.

Linkage Analysis

Based on the location of the gene, the research team designed primers to amplify STR markers linked to the *AR* gene. DXS1194 and DXS8111, two markers linked to the Xq12 region, were chosen. Their relative distances to the target gene were 1.35 Mb and 1.73 Mb toward the centromere and telomere of chromosome X, respectively. The STR amplification primer sequences are as follows: DXS1194f: 5'-/6-FAM/-ACACAACTTGAAACTGCTGAG-3'; DXS1194r: 5'-AAGTATGTTGCCACAGAAACC-3'; DXS8111f: 5'-/6-FAM/- GTAGGAACAATAAGTTATGCCTTGC-3'; DXS8111r: 5'- AGATTAAAGCCCTGGCG −3'.

Similarly, each 25μL PCR reaction tube has 12.5μL of GoTaq Green Mastermix 2X, 5.5μL of Deion water, 1.0μL of each forward and reverse primer, and 5.0μL of template DNA. The Gradient-PCR reaction is carried out using the primer annealing temperature range of 50°C to 60°C, and the ideal primer annealing temperature is chosen as 56°C. The STR amplification thermal cycle is as follows: 98°C - 2 minutes, 25 cycles (95°C - 30 seconds, 56°C - 30 seconds, 72°C - 30 seconds) and 72°C - 10 minutes.

The STR amplification product was mixed with the GeneScan LIZ 500 standard ladder and denatured with Hi-di solution before running capillary electrophoresis on the SeqStudio Genetic Analyzer. GeneMapper ID 6.0 software was used to analyze the results.

Next-Generation Sequencing Analysis

Conduct preimplantation genetic diagnosis for embryos based on the results of the performed Sanger sequencing and linkage analysis techniques. Next, embryo that did not have any mutated allele would be screened for chromosomal abnormalities by Next-Generation Sequencing system (Ion Torrent) using Ion ReproSeq™ kit for PGT-A.

The flowchart of the study protocol was shown in Figure 1.

Results

Embryo's Sex Determination Result

According to the electrophoresis data, a 470 bp band corresponding to the *SRY* gene segment the researchers constructed indicated that the affected child and embryo A1 sample was male (Figure 2).

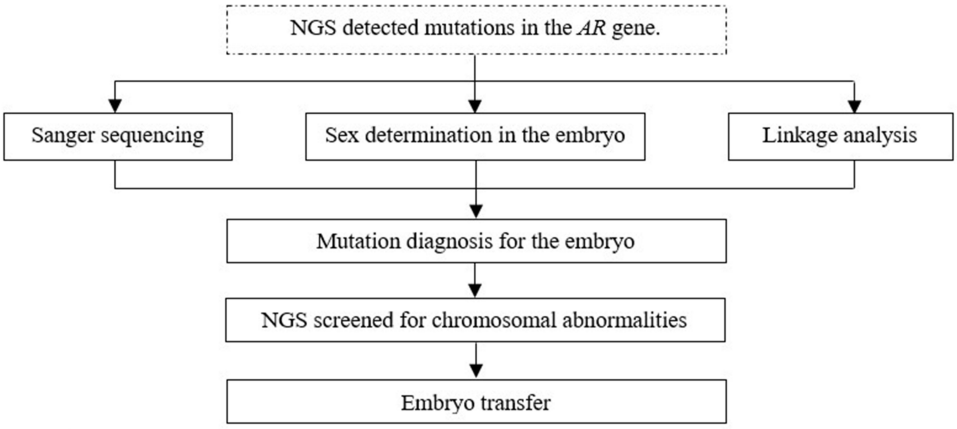

Figure 1 Diagram of preimplantation genetic diagnosis for AIS technique.

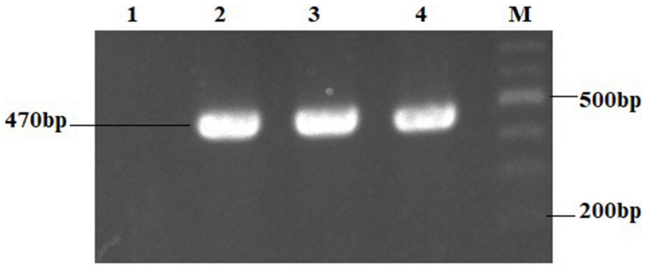

Figure 2 Electrophoresis results of SRY gene amplification. *Land 1: Negative control; Land 2: Positive control; Land 3: Affected child sample; Land 4: Embryo A1 sample; Land (M) Ladder 100bp.*

Results of PCR Reaction and Sanger Sequencing

Using 3% agarose gel electrophoresis (Figure 3) to test the PCR results on the samples revealed that the gene segment with the intended size (276 bp) had been amplified successfully. Sanger sequencing is ensured by the electrophoretic bands, which are bright, clear, and without secondary bands.

Sanger sequencing results (Figure 4) showed that the mother and son samples carried the *AR*:c.796del variant, the father sample was normal and did not carry any mutated genes. The embryo sample shows that the A1 embryo is normal, not carrying the *AR*:c.796del variant. Table 1 provides a summary of the diagnostic results.

Linkage Analysis Results

We analyzed the cases of the mother (I-2) and the son (II-1) based on the capillary electrophoresis results. The alleles linked to the mutant gene are identified as the 285 bp allele of locus DXS1194 (Figure 5A) and the 182 bp allele of locus DXS8111 (Figure 5B). Through genetic linkage analysis on the A1 embryo sample, conclusions can be drawn in Figure 6.

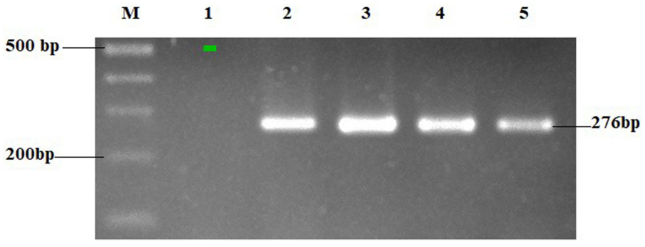

Figure 3 Electrophoresis results of AR gene amplification. Land 1: Negative control; Land 2: Father sample; Land 3: Mother sample; Land 4: Affected child sample; Land 5: Embryo A1 sample; Land (M) Ladder 100bp.

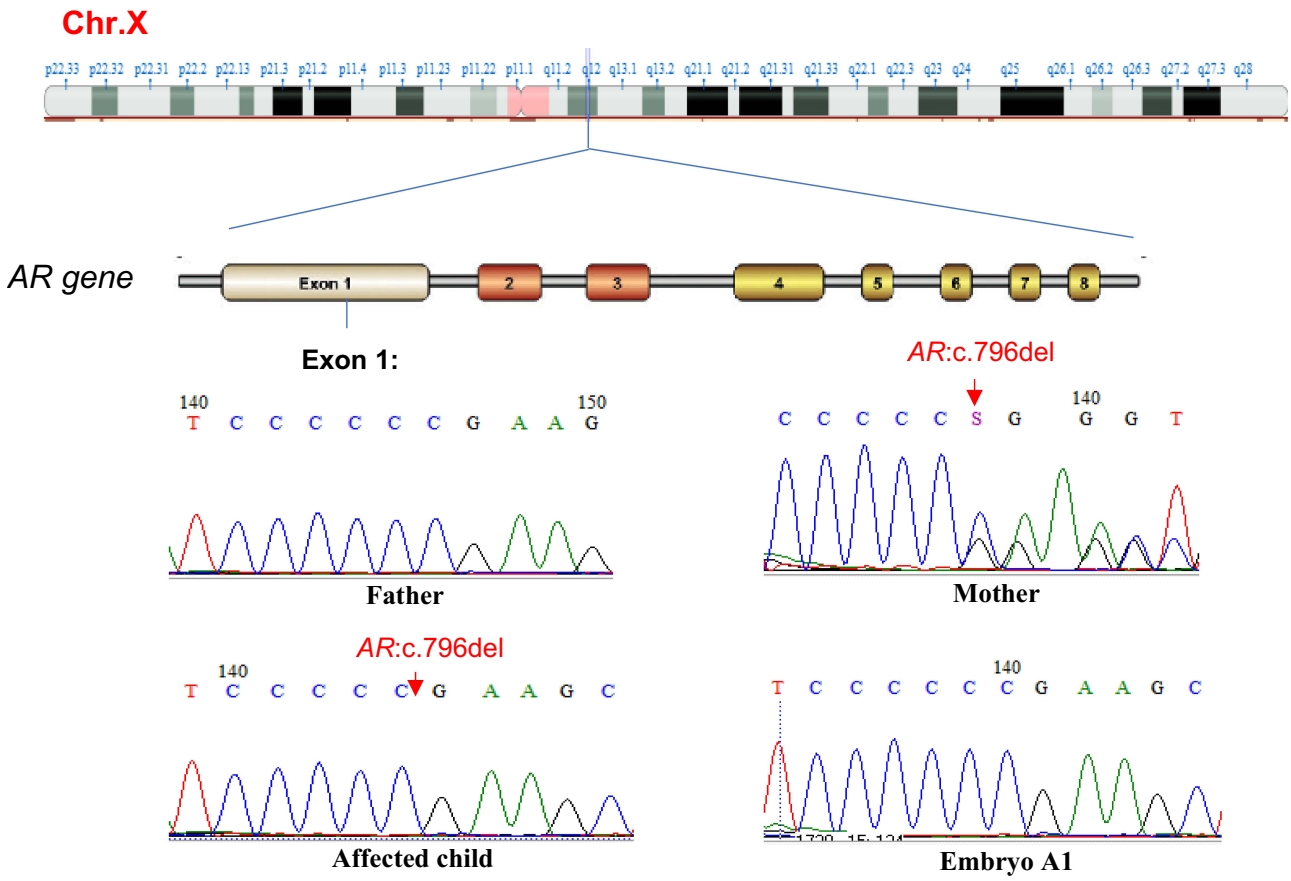

Figure 4 Sanger sequencing results.

The outcomes of Sanger sequencing and linkage analysis were consistent. Thus, the preimplantation genetic diagnosis results the research team implemented are reliable. DNA from embryo A1 reflected a normal embryo and does not carry the mutant gene.

NGS Result

DNA samples of embryo A1, which do not carry mutant allele, will be sequenced to screen for detecting chromosomal abnormalities. NGS screening for the embryo without AIS revealed a normal result, as shown in Figure 7.

Consequently, we concluded that embryo A1 was acceptable and could be transferred to the mother's uterus.

Discussion

The *AR* gene is located on the long arm of the X chromosome, with a coding sequence region of up to 2757 nucleotides, including eight exons and seven introns. The gene that codes for the AR protein has four functional domains in its

Table 1 Summary of the Diagnostic Results Followed Sanger Sequencing Method

Sample	*SRY*	*AR*:c.796	Result
Father	+	WT(G)	Normal
Mother		WT(G)/DelG	Carrier
Affected child	+	DelG	Affected
Embryo A1	+	WT(G)	Normal

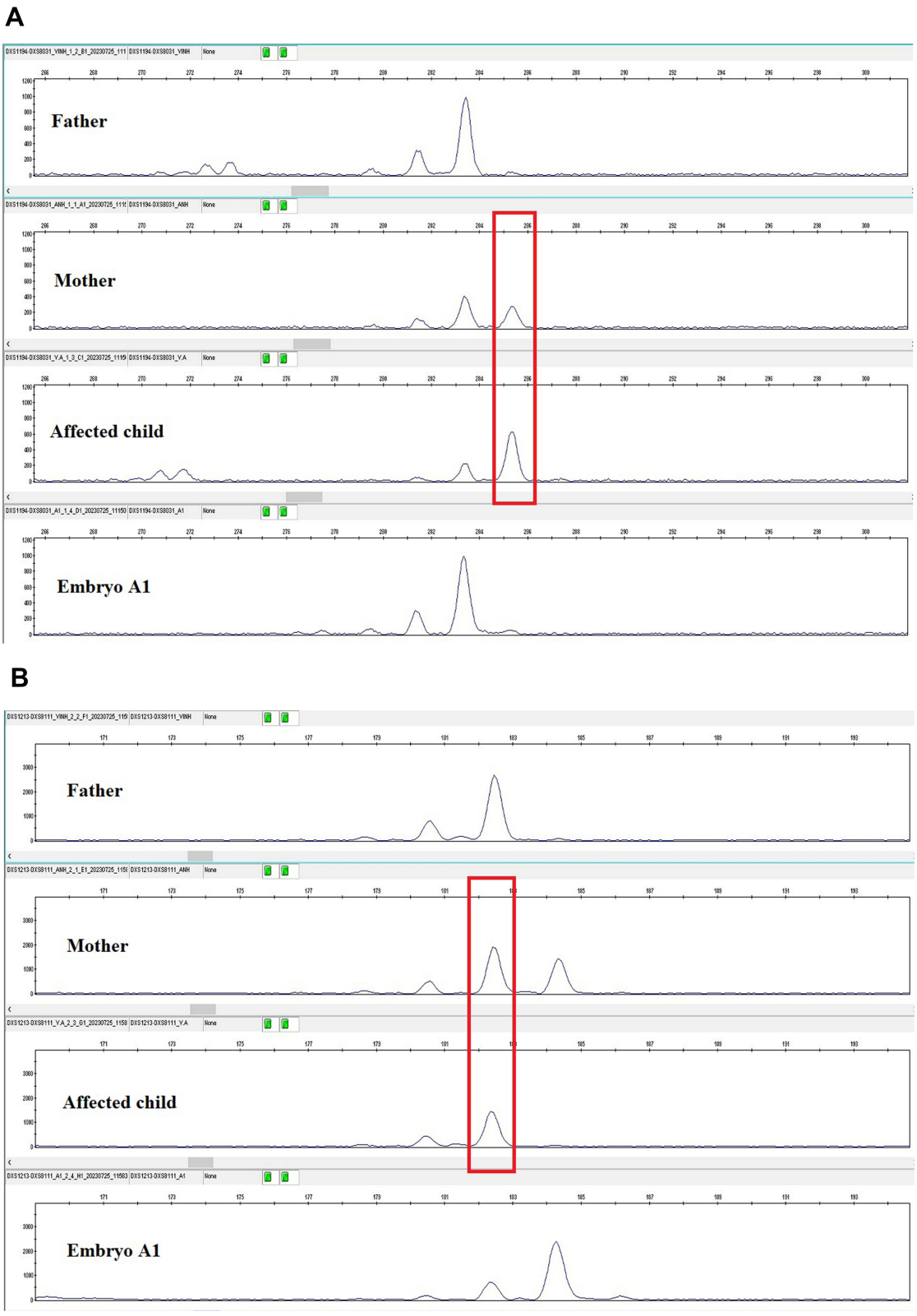

Figure 5 (**A**) Capillary electrophoresis results of DXS1194. *The red-bordered box represents the STR alleles linked to the mutant allele of the AR gene*. (**B**) Capillary electrophoresis results of DXS8111. The red-bordered box represents the STR alleles linked to the mutant allele of the AR gene.

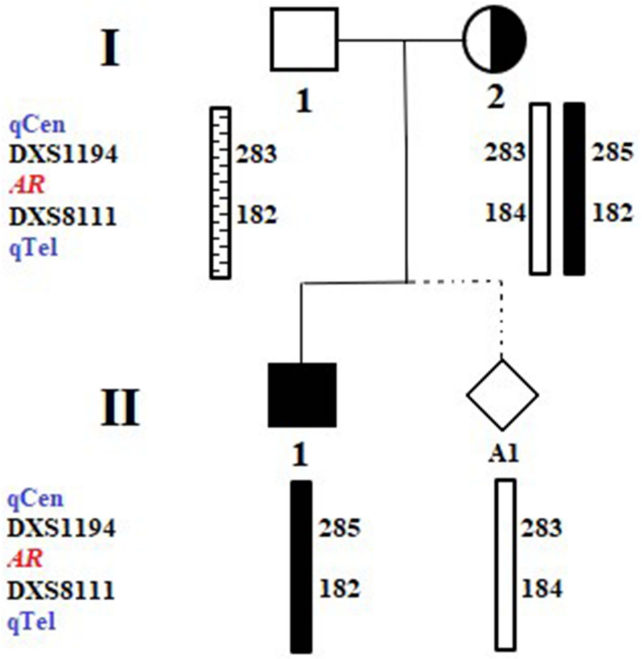

Figure 6 Linkage analysis results.

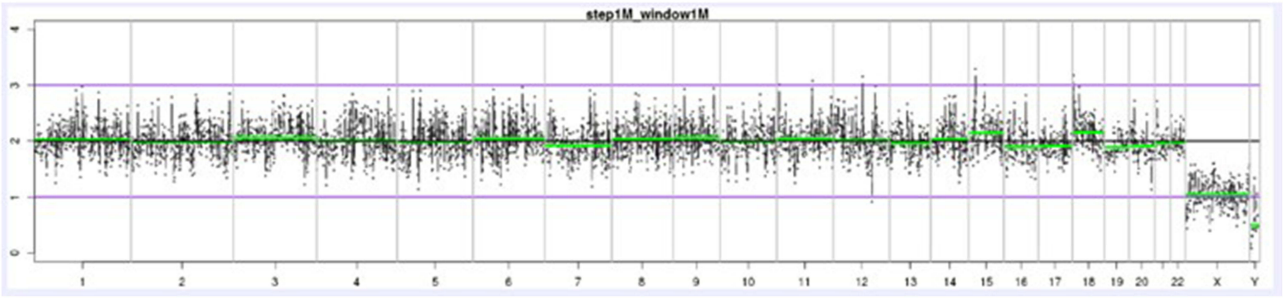

Figure 7 NGS screening aneuploidy result.

structure: The N-terminal domain (NTD), which is encoded by exon 1, is involved in the transcription process that activates target genes; exons 2 and 3 encode the eight central DNA-binding domain (DBD), which has a zinc finger structure; exon 4 encodes the hinge domain; and exons 5 to 8 encode the C-terminal ligand-binding domain (LBD) (Figure 8). The most variable portion of the steroid hormone nuclear receptor family is the DBD, whereas the most highly conserved region is the AR's N-terminal domain.[10,11]

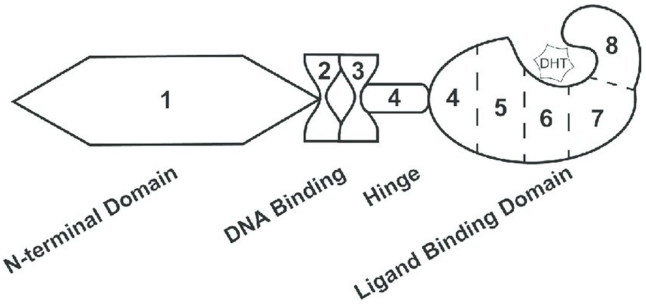

Figure 8 Androgen receptor protein structure and the corresponding exons code for each part.
Notes: Reprinted from Wadosky KM, Koochekpour S. Androgen receptor splice variants and prostate cancer: From bench to bedside. *Oncotarget*, 2017;8(11):18550. Creative Commons.[12]

Currently, up to 406 variants in the *AR* gene are reported as pathogenic and possibly pathogenic on the Clinvar-NCBI database system, of which the majority are missense variants. About 30% of AR gene mutations are de novo and not inherited from previous generations. These mutations stem from germline or gonadal germ cell mosaicism occurring in either parent[13] Variant NM_000044: c.796del (p.Asp266IlefsTer30) discovered above has never been published on databases such as Clinvar (NCBI), 1000 Genome database (1000G) and Exome Aggregation Consortium (ExAC). The loss of the 796th nucleotide in the *AR* gene (DelG) results in a frameshift mutation, causing a change from the 266th amino acid position onwards and premature protein chain termination (at a position 30 amino acids away). The truncated protein is predicted to be synthesized by the gene containing the above variant, including only the amino acids encoded by exon 1, with a length of 295 amino acids (compared to the normal protein chain of 921 amino acids), resulting in a loss of function of AR protein, causing appropriate clinical manifestations in this affected child. However, the limitation of the study is that it cannot confirm whether the protein produced by the *AR* gene carrying this variant is truncated and loses function. Here, our results only show clinical phenotypic concordance in male individuals carrying this variant.

In the literature, there have been reports on CAIS patients carrying variants causing a premature translational stop signal in the *AR* gene. These variants are expected to result in disrupted synthesis of protein products, loss of function and are thought to be pathogenic.[14] On the Clinvar-NCBI database, similar to our variant, a deletion variant leading to premature transcription cessation was published as NM_000044.6(AR):c.1605del (p.Pro534_Tyr535insTer). The *AR* c.1605del variant is located in exon 1 and introduces a premature termination codon at codon 535, predicted to result in non-sense mediated decay. Clinvar classifies this loss-of-function variant in *AR* gene as pathogenic.[15] These data can contribute to increasing confidence in the variant we discovered.

Preimplantation genetic diagnosis of AIS is significant in disease prevention, thereby eliminating mutations in the next generations of AIS families. In this study, based on a case of a family carrying the mutation on *the AR* gene, we performed a preimplantation genetic diagnosis of AIS by combining Sanger sequencing and linkage analysis using STR to minimize the risk of misdiagnosis due to amplification failure, biased amplification, allele dropout and sample contamination.[16,17] Linkage analysis can be considered as an indirect method to check Sanger sequencing results. In addition, in preimplantation genetic diagnosis of sex-linked genetic diseases, determining the gender indicator locus is essential (in this research, we used the *SRY* gene)[18] because the characterize of carrying only one X chromosome on male embryo will cause difficulty and confusion in determining whether the allele drop-out phenomenon occurs in female embryos carrying the homozygous genotype or not.

Preimplantation genetic diagnosis of AIS has significant value in disease prevention. In the case reported above, we successfully carried out the preimplantation genetic diagnosis for an embryo of a family carrying the variant NM_000044: c.796del (p.Asp266IlefsTer30), results: 01 normal embryo, does not carry this variant and can be used to perform embryo transfer. The same method used in the study can be applied to preimplantation genetic diagnosis in additional clinical instances with *AR* mutations.

Conclusion

We report on a novel variant in the *AR* gene discovered in Vietnam. In addition, we have developed the preimplantation genetic diagnosis for AIS and applied the technique to an embryo from a couple who had previously had an affected son. The result: a normal embryo, not carrying the variant NM_000044: c.796del (p.Asp266IlefsTer30).

Abbreviations

NGS, Next-generation sequencing; STR, Short tandem repeats; AIS, Androgen Insensitivity Syndrome; CAIS, Complete androgen insensitivity syndrome; PAIS, Partially androgen insensitivity syndrome; IVF, In vitro fertilization.

Data Sharing Statement

The corresponding author will provide the data supporting the study's conclusions upon a reasonable request. In case you have concerns regarding data sharing, please write to trieusangk83@yahoo.com.vn.

Ethical Statements

Each participant in the research voluntarily agreed to participate after receiving an adequate explanation of the study's purpose and risks. In order to publish the case, participants provided written informed consent, and the procedure was approved by the Ethical Review Committee of Vietnam Military Medical University (No.1068/2019/VMMU-IRB). The Declaration of Helsinki, its later revisions, or comparable ethical standards were followed in the conduct of this study, along with appropriate clinical practice.

Consent for Publication

We are aware that The Application of Clinical Genetics journal will be accessible to a broader audience through marketing channels and other third parties and that it may be published in print and online. Consequently, anything published in the journal is accessible to everyone. We know that readers may consist of journalists, members of the public, academic researchers, and medical experts. Please send an Email to trieusangk83@yahoo.com.vn with any questions.

Institutional approval was required to publish the research and case details.

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Author Contributions

The work reported has significant contributions from all authors, who took part in the conception, study design, execution, data acquisition, analysis, interpretation, drafting, revising, and critical review of the article, gave the final version to be published, agreed upon the journal to which the article has been submitted, and agreed to take full responsibility for all aspects of the work.

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Disclosure

Regarding this paper's research, writing, and publishing, the authors have disclosed no potential conflicts of interest.

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