

# Identification of the Underlying Androgen Receptor Defect in the Dallas Reifenstein Family

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**Context:** The Dallas Reifenstein family — first described in 1965 — includes 14 known members with partial androgen insensitivity syndrome (PAIS). However, the underlying molecular defect was never identified.

**Objective:** To identify the underlying genetic defect for PAIS in the Dallas Reifenstein family.

**Design:** DNA was purified from scrotal skin fibroblasts, and whole exome sequencing was then performed in four affected men in the family. Additional family members — both affected and unaffected — were subsequently recruited to confirm segregation of the candidate mutations with the PAIS phenotype.

**Patients:** The affected men have PAIS with infertility associated with azoospermia, hypospadias, and gynecomastia.

**Results:** All four men harbored an intronic variant NC\_000023.10:g.66788676A>C between exon 1 and exon 2 of the androgen receptor (*AR*) canonical transcript NM\_000044 (complementary DNA position NM\_000044: c.1616+22072A>C) predicted to cause an alternatively spliced *AR* transcript. Reverse transcription (RT) polymerase chain (PCR) experiments detected the predicted PCR product of the alternatively spliced *AR* transcript, and the mutation segregated with the PAIS phenotype in this family. The transcript includes the insertion of 185 nucleotides with a premature stop codon at chrX:66863131-66863133, likely resulting in a reduction in *AR* protein expression due to nonsense-mediated decay.

**Conclusions:** An intronic *AR* mutation was identified in the Dallas Reifenstein family. The findings suggest that in cases of PAIS without identifiable *AR* mutations in coding regions, intronic *AR* mutations should be considered.

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**Freeform/Key Words:** Reifenstein, androgen receptor, androgen insensitivity syndrome, hypospadias, PAIS, *AR*

Hereditary resistance—described for almost all hormones—is most commonly due to dysfunctional hormone receptors, and the most frequent of these disorders involves resistance to androgen action [1]. The high incidence of androgen resistance is due to at least two factors.

Abbreviations: *AR*, androgen receptor; cDNA, complementary DNA; mRNA, messenger RNA; PAIS, partial androgen insensitivity syndrome; PCR, polymerase chain reaction; RT-PCR, reverse transcription polymerase chain reaction.

First, hemizygous deficiency of the androgen receptor (AR) is sufficient to cause human disease in 46, XY individuals because the *AR* gene is located on the X chromosome [2]. Second, a strong ascertainment bias for diagnosing androgen resistance is common because the action of androgen from the developing testes is critical for development of the male urogenital tract during embryogenesis.

As of 2016, some 500 different loss-of-function mutations have been described in the human *AR* coding region [3]. *AR* mutations vary in severity from complete androgen insensitivity syndrome (genetic males with female phenotypes) to partial androgen insensitivity syndrome (PAIS, genetic males with variable features, such as gynecomastia, hypospadias, infertility, and/or minor defects in virilization).

Interestingly, only 30% of all PAIS cases harbor identifiable *AR* mutations [4]. This conundrum is demonstrated by the Dallas Reifenstein family, originally reported by Bowen *et al.* in 1965 [5]. This family has been studied repeatedly over the years and includes 14 known examples of PAIS associated with male infertility (due to azoospermia), hypospadias, gynecomastia, and inheritance in a manner suggestive of X-linkage [6]. Indeed, formal linkage analysis established that the Dallas Reifenstein family mutation is closely linked to the area encoding the *AR* [7]. However, repeated sequencing of the *AR* coding exons has not revealed any coding mutations in this family, and investigation of cultured skin fibroblasts in such patients has not revealed any qualitative abnormality in receptor function, leading us to conclude that the underlying mutations in this family must influence the amount of receptor rather than its quality [8].

Here we report the use of whole-exome sequencing to identify the responsible mutation in the Dallas Reifenstein family, namely an A-to-C substitution in intron 1 of the *AR* gene, resulting in an alternatively spliced transcript with a cryptic exon.

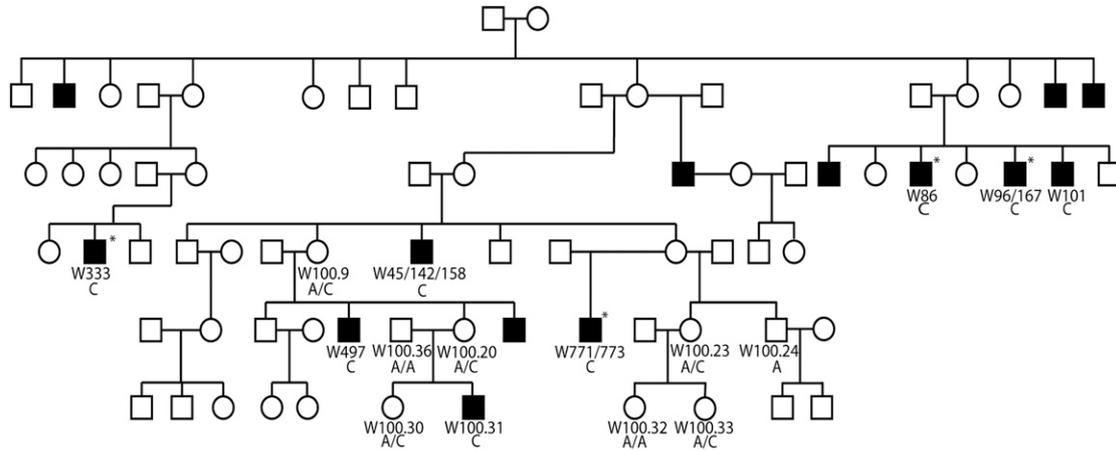
## 1. Patients and Methods

All patients or their guardians gave written informed consent, and the Institutional Review Board of UT Southwestern Medical Center approved the protocol. Scrotal skin fibroblasts from seven affected members (W45/142/158, W86, W96/167, W101, W333, W497, and W771/773) were obtained during prior investigations and stored in liquid nitrogen [5]. These patients were all previously karyotyped as XY and have been described previously [5, 8]. Briefly, they presented with hypospadias at birth and developed gynecomastia during puberty. A few also presented with bifid scrotum at birth, and several had undergone testicular biopsies that revealed sparse or absent Leydig cells [5, 8].

In addition, we recruited 10 other members of the family, including a 14-month-old boy (W100.31) born with ambiguous genitalia (Fig. 1). The remaining nine newly ascertained relatives were healthy. The 14-month-old boy was born with perineoscrotal hypospadias. On physical examination, he had no dysmorphic facial features, 1.5-mL testes were palpable bilaterally in scrotal tissue, and his stretched penile length was 2.3 cm (width of glans, 0.8 cm). His testosterone level (checked at age 5 weeks) was 647 ng/dL (normal, 75 to 400 ng/dL), follicle-stimulating hormone level was 1.12 mIU/mL (normal, 0.16 to 4.1 mIU/mL), and luteinizing hormone level was 6.7 mIU/mL (normal, 0 to 0.26 mIU/mL). Renal ultrasonography showed normal kidneys, and pelvic ultrasonography confirmed the presence of gonadal tissue within the scrotum and no evidence of a uterus. Fluorescence *in situ* hybridization showed normal XY chromosomes. At 6 months of age, the boy successfully underwent surgical repair of the hypospadias.

### A. Whole-Exome Sequencing

DNA was purified from scrotal skin fibroblasts [5], and whole-exome sequencing was performed on four affected men (W86, W771/773, W333 and W96/167). Libraries of samples were prepared by using the SureSelect Human All Exon V4 kit (Agilent, Santa Clara, CA). Sequencing was run on an Illumina HiSeq2500 with the read length of paired-end



**Figure 1.** Pedigree for the Dallas Reifenstein family (adapted and updated from [8]). Shaded boxes indicate affected males. Subject identifiers are listed below each identified individual, and genotype for the intronic mutation NC\_000023.10:g.66788676A>C is listed below the identifier. A, wild-type male; A/A, wild-type females; A/C, heterozygous female carriers; C, male with mutation. \*Samples sent for whole exome sequencing.

2 × 100 bp by the McDermott Center Sequencing Core at UT Southwestern. Sequences were aligned to the human reference genome hg19, and variants were called by using the Genome Analysis Toolkit HaplotypeCaller and annotated by using snpEff.

We paid special attention to variants in the proximity of the *AR* gene because linkage studies suggested regions near *AR* [7], even though prior efforts with Sanger sequencing of the *AR* coding region failed to identify any mutations. Of note, the SureSelect Human All Exon V4kit (Agilent) amplified a large region of intron 1 since this region includes exon 1 of an alternative *AR* transcript NM\_001011645.

### B. Sanger Sequencing

Genomic DNA was isolated from peripheral blood or scrotal skin fibroblasts by using the Easy-DNA kit (ThermoFisher Scientific, Carlsbad, CA). Candidate DNA mutations were identified by amplifying candidate regions with gene-specific primers and Sanger sequenced using ABI Prism 3100.

### C. RT-PCR Experiments

Because we identified an intronic mutation, we performed additional experiments to study the effects of the mutation on gene expression. Total RNA was extracted from fibroblasts of a family member (W771/773) with the intronic variant in the *AR* gene and an unaffected individual by using the RNeasy Mini kit (Qiagen), including an on-column DNase digestion (Qiagen) step according to the manufacturer's instructions. Complementary DNA (cDNA) was synthesized by using the iScript Reverse Transcription Supermix (Bio-Rad). Reverse transcription (RT) polymerase chain reaction (PCR) was performed with MyTaq Polymerase (Bioline) in 50- $\mu$ L reactions with an initial denaturation at 94°C for 3 minutes, followed by 35 cycles of 94°C for 30 seconds, 60°C for 30 seconds, and 72°C for 30 seconds and a final elongation step of 72°C for 5 minutes. Oligonucleotide primers were designed for the PCR amplification of cDNA fragments specific for wildtype *AR* transcripts (5'-GACTTCACCG-CACCTGATGT-3' and 5'-GGAAAACTTACCGCATGTCC-3') and alternatively spliced *AR* transcript (5'-GGTGAGCAGAGTGCCCTATC -3' and 5'-TGCATAGGCAAGAAGATGGA-3'). We used a cDNA fragment of ubiquitously expressed  $\beta_2$ -Microglobulin as a positive control with the oligonucleotide primers 5'-TGACTTTGTCACAGCCCAAG-3' and 5'-AGCAAGCA-AGCAGAAATTTGG-3'. PCR products were separated on a 3% agarose gel.

## 2. Results

The whole-exome sequencing mean coverage of samples W86, W771, W333, and W96 was 113, 118, 166, and 146, respectively. All four participants harbored an intronic variant NC\_000023.10:g.66788676A>C between exon 1 and exon 2 of the *AR* canonical transcript NM\_000044 (cDNA position NM\_000044: c.1616+22072A>C).

Sanger sequencing confirmed this variant in the seven affected men whose DNA was extracted from stored scrotal skin fibroblasts (W45/142/158, W86, W96/167, W101, W333, W497, W771/773). Sanger sequencing was also performed in the 10 newly recruited family members, and the 14-month-old boy (W100.31) with ambiguous genitalia also had the mutation. The mother (W100.20) and grandmother (W100.9) of the boy—predicted to be obligate carriers—were both heterozygous for the mutation, whereas an unaffected male relative (W100.24) lacked the variant. In summary, the variant cosegregated with the PAIS phenotype in this family.

This mutation is located in a sequence region that conforms well to splice-site consensus sequences. It is a purine (A) to pyrimidine (C) substitution at the end of a polypyrimidine tract 2 bp upstream of the canonical splice acceptor site AG. We performed *in silico* analysis by using SplicePort [9], which makes splice-site predictions based on a collection of 4000 pre-messenger RNA (mRNA) human RefSeq sequences using a feature generation algorithm. The result suggested that this substitution creates a cryptic exon of 185 nucleotides between exons 1 and 2 of the canonical *AR* transcript NM\_000044 by fusing with exon 1 of an alternative *AR* transcript NM\_001011645 [Fig. 2(a)].

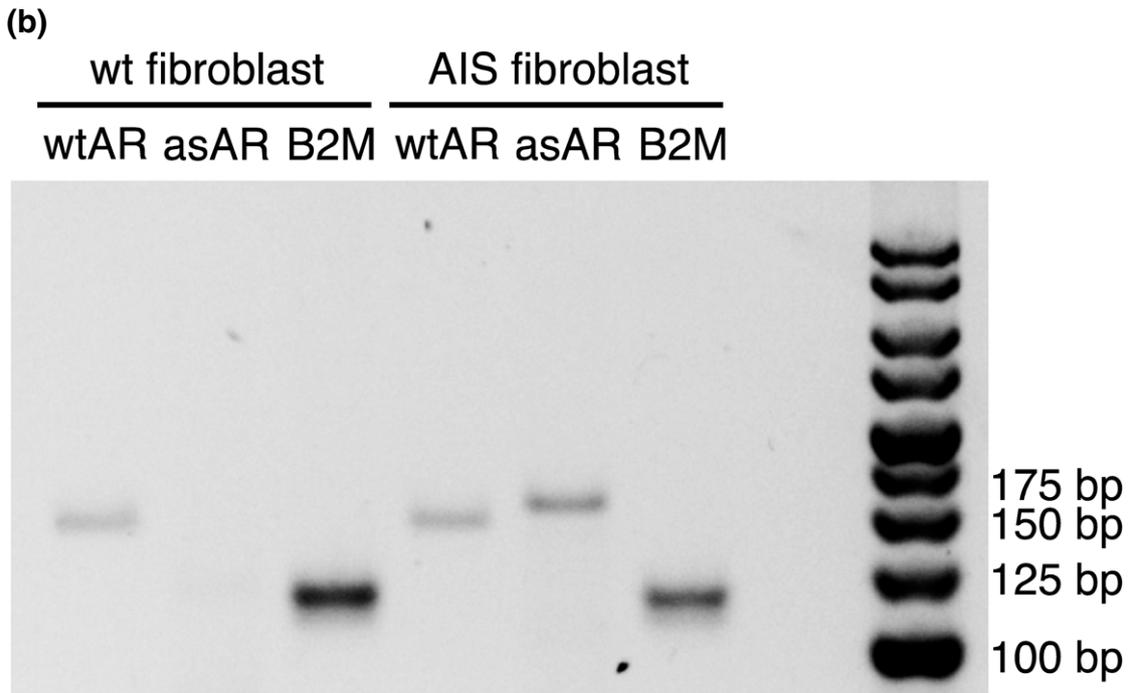
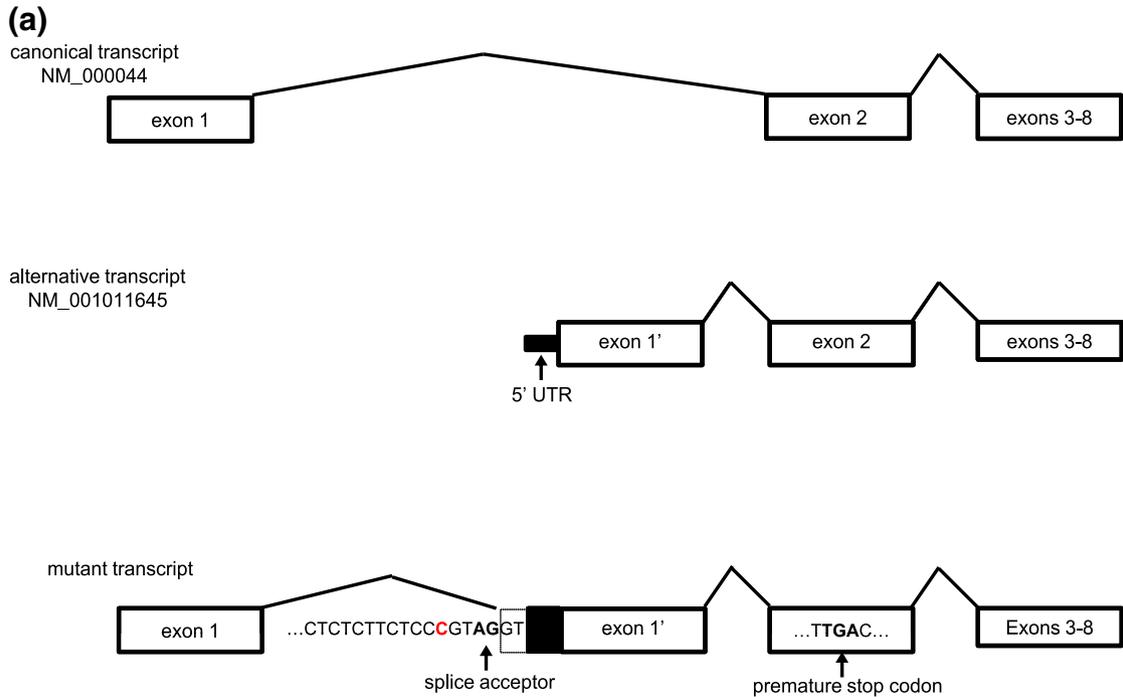
We tested for the presence of this *AR* transcript by RT-PCR in a fibroblast preparation from an unaffected individual and an individual (W771/773) with the mutation. For this RT-PCR assay, we designed primers that specifically amplify a fragment of the wild-type *AR* cDNA (wtAR) or the alternatively spliced *AR* cDNA. We detected the predicted PCR product of the alternatively spliced *AR* transcript only for the cDNA of the individual with the mutation [Fig. 2(b)]. The sequence of this PCR product was validated by Sanger sequencing, indicating that this mutation causes the expression of an *AR* transcript in carriers. The insertion of 185 nucleotides would result in the expression of an alternatively spliced *AR* transcript with a premature stop codon at chrX:66863131-66863133 and consequently a reduction in *AR* protein expression due to nonsense-mediated decay.

## 3. Discussion

By using whole-exome sequencing and focusing on noncoding regions, we identified a causative *AR* mutation in a historic cluster of patients with PAIS—the Dallas Reifenstein family. The causative mutation occurs in intron 1 of the *AR* gene and leads to alternative splicing, presumably resulting in nonsense-mediated decay [10]. Although further studies may be needed to confirm the hypothesis about nonsense-mediated decay, our interpretation is consistent with prior studies in this family suggesting a 50% reduced quantity of otherwise functional *AR* [8].

*AR* intronic mutations represent <3% of the mutations in patients with currently recognized androgen insensitivity syndrome, and almost all prior reports are of canonical splice-site mutations close to exon-intron junctions [3, 11, 12]. Similar to our findings, however, Brüggewirth *et al.* [13] described a mutation in intron 2 in a family with three individuals with PAIS. Sequencing analysis showed the mutation involves a T-to-A substitution 11 bp upstream of exon 3. The location of the mutation preceded the splice-acceptor site, leading to aberrant splicing by activating a cryptic splice site and ultimately resulting in transcripts encoding defective ARs [13]. Most recently, Käsäkoski *et al.* [14] performed whole-genome sequencing in 46, XY sisters with complete androgen insensitivity syndrome and identified a mutation deep in intron 6 of *AR*. The mutation caused aberrant splicing of *AR* mRNA, creating two abnormally long products and a significantly reduced amount of the normal-sized mRNA.

The ability to identify underlying genetic causes in disorders of sexual development has several clinical implications: establishing a definitive diagnosis, providing more accurate



**Figure 2.** (a) Illustration of wild-type and aberrant splicing process resulting from the intronic mutation. The nucleotide in red is the NC\_000023.10:g.66788676A>C mutation. *In silico* analysis suggested that this substitution creates a cryptic exon of 185 nucleotides between exons 1 and 2 of the canonical AR transcript NM\_000044 by fusing with exon 1' of an alternative AR transcript, NM\_001011645. (b) RT-PCR detects an AR transcript in a patient with androgen insensitivity syndrome with an AR intronic mutation (W771/773). RT-PCR was performed for cDNA generated from RNA of fibroblast from an unaffected individual (wt) and an individual with androgen insensitivity syndrome with an intronic single nucleotide variant in the AR gene. PCR products were separated on a 3% agarose gel. asAR, RT-PCR product from alternatively spliced AR transcript that results from the single nucleotide variant; B2M, RT-PCR product from  $\beta_2$ -microglobulin transcript (positive control); UTR, untranslated region; wtAR, RT-PCR product from canonical (or wild-type) AR transcript.

genetic counseling (especially for female carriers), and allowing for potential prenatal screening [15]. In addition, prognosis may be different according to whether a PAIS mutation is identified. Data from the International Disorders of Sex Development Registry suggests that boys with genetically confirmed PAIS are likely to have poorer clinical outcome (*e.g.*, multiple surgeries) than those without a detectable mutation [16].

Whole-exome sequencing has dramatically accelerated the pace of elucidating novel, disease-causing genes, in particular, for rare Mendelian disorders [17]. However, the overall diagnosis rate remains low [18]. One possible explanation is that pathogenic mutations lie in noncoding regions more frequently than currently recognized.

Although the disease-causing mutation we identified is embedded deep in intron 1 of the *AR* canonical transcript NM\_000044, it is in close proximity to exon 1 of the *AR* alternative transcript NM\_001011645, which is a targeted region of the SureSelect kit; this allowed us to capture this mutation.

This study indirectly demonstrates the potential importance of using whole-genome sequencing to identify genetic causes of diseases. For example, whole-genome sequencing was essential in dissecting X-linked reticulate pigmentary disorder [19].

Alternatively, a custom next-generation sequencing kit could be the recommendable technique specifically for *AR* intronic mutations if the kit includes the introns of the *AR* gene. Such a kit has been described: Haloplex protocol (Agilent) includes oligos spanning the chromosomal region ChrX:66,754,874–66,955,461 (hg19) [20, 21].

In summary, we identified a disease-causing intronic mutation in the Dallas Reifenstein family, a pedigree that includes several generations of men affected by PAIS. Our findings suggest that noncoding regions of *AR* should be suspected when no mutations can be found in coding regions.

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Disclosure Summary: The authors have nothing to disclose.

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