Novel Insights from Clinical Practice

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An Intron 9 CYP19 Gene Variant (IVS9+5G>A), Present in an Aromatase-Deficient Girl, Affects Normal Splicing and Is Also Present in Normal Human Steroidogenic Tissues

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Established Facts

- Aromatase, the enzyme complex that catalyzes the synthesis of estrogens from androgens, undergoes different alternative splicing events in the coding region that may lead to both active or inactive proteins in normal and/or in aromatase deficiency tissues.

Novel Insights

- A novel IVS9+5G>A mutation generating a splicing variant that includes intron 9 was found. This was also present in the absence of the mutation in normal steroidogenesis tissues, suggesting that a misbalance between normal and aberrant splicing variants might explain the partial deficiency phenotype.

Key Words
Aromatase deficiency · Mutation · *CYP19A1* · Steroidogenic tissues · Splicing

Abstract
Background/Aims: Splicing CYP19 gene variants causing aromatase deficiency in 46,XX disorder of sexual development (DSD) patients have been reported in a few cases. A misbalance between normal and aberrant splicing variants was proposed to explain spontaneous pubertal breast development but an incomplete sex maturation progress. The aim of this study was to functionally characterize a novel *CYP19A1* intronic homozygote mutation (IVS9+5G>A) in a 46,XX DSD girl presenting spontaneous breast development and primary amenorrhea, and to evaluate similar splicing

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variant expression in normal steroidogenic tissues. ***Methods:*** Genomic DNA analysis, splicing prediction programs, splicing assays, and in vitro protein expression and enzyme activity analyses were carried out. *CYP19A1* mRNA expression in human steroidogenic tissues was also studied. ***Results:*** A novel IVS9+5G>A homozygote mutation was found. In silico analysis predicts the disappearance of the splicing donor site in intron 9, confirmed by patient peripheral leukocyte cP450arom and in vitro studies. Protein analysis showed a shorter and inactive protein. The intron 9 transcript variant was also found in human steroidogenic tissues. ***Conclusions:*** The mutation IVS9+5G>A generates a splicing variant that includes intron 9 which is also present in normal human steroidogenic tissues, suggesting that a misbalance between normal and aberrant splicing variants might occur in target tissues, explaining the clinical phenotype in the affected patient.

Introduction

Aromatase is the enzyme complex that catalyzes the synthesis of estrogens from androgens. The biological importance of the aromatase complex is related not only to its role in the synthesis of estrogens, but also to its potential influence on the balance of the androgen-estrogen ratio in different tissues. In humans, cP450arom appears to be the product of a single gene *(CYP19A1)* that is localized in chromosome 15q21.1. cP450arom is expressed in a number of tissues, including the syncytiotrophoblast layer of the placenta, gonads, adipose tissue, bone, brain (including the hypothalamus, hippocampus, and amygdala), coronary arteries, and various fetal tissues, such as liver, skin, intestine, testis, and ovary. The protein coding sequence is included in nine exons (exons 2–10) which span approximately 35 kb. The translation initiation and termination codons are present in exon 2 and exon 10, respectively. Although there are multiple first exons involved in tissue-specific expression, it has been described that the protein sequence is conserved [1–3].

Aromatase deficiency is a rare autosomal recessive disorder in which affected patients cannot synthesize estrogens normally. Fetuses lacking aromatase activity are not able to convert DHEA-S produced by the fetal adrenal gland to estrogens in the placenta; DHEA-S is therefore converted to testosterone, resulting in the virilization of both fetus and mother. Since the first description of aromatase deficiency by Shozu et al. [4] in 1991, 28 cases have been reported (18 females and 10 males) [5–11].

Herein, we report a novel splicing site intronic homozygous mutation (IVS9+5G>A) in the *CYP19A1* gene, localized five bases downstream of the 5′ beginning of intron 9, in a 16.4-year-old Brazilian girl suspected of having an aromatase deficiency. Functional studies revealed an aberrant mRNA splicing, resulting in the retention of intron 9 that translates to a shorter inactive protein losing the heme-binding site. However, the girl showed spontaneous breast development and pubertal estradiol (E_2) levels during follow-up, suggesting residual P450arom activity.

Alternative splicing events in the coding region of the P450arom RNA that lead to putative inactive proteins have been described in several species, including higher primates [12–17]. It has been suggested that these alternative splicing events may be another essential mechanism, among paracrine and endocrine factors, to control P450arom expression, thereby regulating estrogen production [14]. In the rat ovary, three P450arom mRNAs have been detected, two of them lacking exon 10, which encodes for the heme-binding domain [18]. In the ovary of the Medaka fish, two splicing cDNA variants were found, one of which also lacks the sequence coding for the heme-binding domain as a result of a frameshift in the open reading frame, the consequence of an additional nucleotide [19]. In rabbits, two aromatase transcript variants of 2.9 and 1.5 kb have been described [15]. The 1.5-kb variant is identical to the 2.9-kb variant at its 5′ end but differs at its 3′ end downstream of nucleotide 1384. The truncated variant includes a short specific sequence of unspliced intron 9 instead of exon 10. The absence of exon 10 might be the consequence of the alternative use of a polyadenylation signal found in the 5′ end of intron 9, at base 1433. The presence of an in-frame stop codon (TAA) 18 bp downstream of the splice junction in intron 9 produces a shorter protein of an apparent molecular mass of 46 kDa in rabbit preovulatory granulosa cells [20].

We previously described the expression of an alternatively spliced mRNA lacking exon 5 (–Ex5) in normal human steroidogenic tissues that would be translated to an inactive protein. We suggested that in normal human tissues this alternative splicing event (–Ex5) might be a physiological regulatory mechanism for estrogen synthesis [21].

In this study, we describe, in normal human steroidogenic tissues, the presence of an alternative splicing event in the coding sequence of P450arom that would retain intron 9 (P450arom-In9) and that would translate to a shorter and inactive protein. We hypothesize that the

presence of the IVS9+5G>A mutation might affect the natural balance of alternative splicing events increasing the intron 9 variant transcription. However, some transcripts that would translate into the 53-kDa active protein might be expressed, explaining the spontaneous breast development and pubertal E_2 found in the patient.

Materials and Methods

Case Report and Clinical Follow-Up

The affected 46,XX patient was reportedly born to nonconsanguineous parents after an uncomplicated pregnancy. No sign of virilization was reported in the mother. From birth to 3 years of chronological age (CA), she was treated with glucocorticoids and fludrocortisone. Laboratory work-up excluded congenital adrenal hyperplasia. She was referred to the Pediatric Endocrinology Unit at 4 years of age presenting labioscrotal fusion, clitoromegaly (1.5 cm long), and a perineal urethra; normal ovaries and uterus were detected by ultrasound. At 4.9 years of CA, she was submitted to surgical correction of the external genitalia. At 13.5 years of CA, she presented a delayed bone age (10 years [22]), a height of 139 cm (height SDS –1.9), a weight of 38 kg (weight SDS –0.65), Tanner stage IV pubic hair, and Tanner stage 1 breasts (online suppl. table 1; for all online suppl. material, see www.karger.com/doi/10.1159/000437142). Pelvic ultrasound revealed a small uterus and the presence of follicular cysts in both ovaries; laboratory findings showed elevated basal serum LH and FSH levels and early-puberty basal serum levels of estrogens (27.7 pg/ml). Puberty was induced with conjugated estrogens, and subsequently progestogens were added. Menarche was induced at 15 years of age. At 16.4 years of CA, her height was 157.5 cm (height SDS –1.0; midparental height SDS –2.2). She had delayed bone age (12.5 years) and low bone mineral density (z-scores –1.8 at L1–L4 and –2.1 at L4; online suppl. fig. 1). Pelvic ultrasound showed enlarged ovaries and multiple follicular cysts. Elevated basal serum LH and FSH levels were detected. Aromatase deficiency was suspected. At 17.3 years of age, she was given Q-Laira® (Bayer), a continuous-use combined oral contraceptive providing cycling differential estrogen concentrations.

The present study was approved by the Ethical Committee of the Federal University of Parana Clinical Hospital and the Institutional Review Board of Garrahan Pediatric Hospital, and informed consent was obtained from the parents and the patient.

DNA Isolation, Amplification, and Sequencing

Genomic DNA was isolated from peripheral leukocytes (PBLs) of the affected subjects and relatives using standard techniques. Each coding exon (2–10) of the *CYP19* gene was amplified and sequenced as previously described [23].

RNA Isolation and Reverse Transcriptase-Polymerase Chain Reaction Assay

Total RNA was isolated from PBLs, transfected and nontransfected Y1 or COS-7 cells, BeWo and H295R cells, and tissues were homogenized (Ultra-Turrax T25 homogenizer) using TRIzol reagent (Invitrogen) according to the manufacturer's instructions. The purity and integrity of each total RNA sample were evaluated using spectrophotometry and gel electrophoresis. Five micrograms of total RNA were reverse transcribed with Moloney murine leukemia virus reverse transcriptase (M-MLV-RT). RT products from PBLs and Y1 cells were used as polymerase chain reaction (PCR) templates with primers corresponding to exon 8 (5′-cgtggtgacctgacaagaga-3′) and exon 10 (5′-gggtaaagatcatttccagca-3′) and primers corresponding to exon 8 (5′-gaagaggcaataataaaggaaatc-3′) and intron 9 (5′-aacacaaagaaccagagagg-3′) amplifying a PCR fragment of 633 and 358 bp, respectively. RT products from human steroidogenic tissues and human cell lines were PCR amplified using primers amplifying exon 8 to intron 9 (358-bp fragment) or primers amplifying exon 9 (5′-gaatattggaaggatgcacagact-3′) to exon 10 (5′-gggtaaagatcatttccagcatgt-3′; 293-bp fragment).

In order to avoid genomic DNA amplification, primers were chosen to bind over different exons. Purified amplicons were used as templates for direct sequencing. As normal control, total RNA was extracted from PBLs of a donor not presenting endocrine or metabolic disease. Total RNA extracted from placental tissues was used as a positive control.

Minigene Construction

The constructs were made by the sequential cloning of 3 PCR fragments containing part of exons 8 and 10, full-length exon 9, and each flanking intronic sequences into pTARGET (Promega) or pcDNA3 (Invitrogen) mammalian expression vectors (online suppl. fig. 2). Mutant (Mut) minigene was obtained by replacing the fragment of exon 9 in the wild-type (WT) minigene with a PCR fragment containing the mutation obtained from the patient's DNA. Sequences of minigenes were verified by direct sequencing.

Construction of p-AroIn9Mut

The P450aro expression vector (p-Aro) previously constructed by Pepe et al. [21] was used as a template to generate the P450aromIn9mut expression vector (p-AroIn9Mut) by enzymatic digestion and replacement of the exon 9 to exon 10 sequence for the exon 9 to intron 9 sequence. Briefly, a fragment containing part of exon 9 to the first 458 nucleotides of intron 9 was amplified from patient genomic DNA by PCR with specific primers (5′-tgccagtttgctcgtaagtg-3′ and 5′-atgggacctactgctcatgg-3′). Both p-Aro and the PCR fragment were digested with XhoI and ApaI, purified, and, finally, they were ligated. The p-AroIn9Mut construct sequence was verified by direct sequencing.

Cell Culture and Transfections

Mouse adrenal Y1 cells (ATCC CCL-79) were maintained in six-well plates in DMEM/F12 (Gibco) supplemented with 10% FBS. Cells were transiently transfected with WT or Mut minigenes using Lipofectamine 2000 (Invitrogen) for splicing assays. Forty-eight hours after transfection, RNA was isolated and RT-PCR was performed. Each transfection experiment was repeated at least three times.

COS-7 cells were maintained in six-well plates in DMEM-HG (Gibco) supplemented with 10% FBS. Cells were transiently transfected with a p-Aro or p-AroIn9Mut using Lipofectamine 2000 (Invitrogen). Forty-eight hours after transfection, proteins were isolated and Western blot was performed. Each transfection experiment was repeated at least three times.

Western Blot and Aromatase Activity Assay

Proteins were obtained with TRIzol reagent 48 h after transfection. Fifty micrograms of protein were electrophoresed in 10% SDS-PAGE and electroblotted to PVDF membranes. Aromatase protein was revealed using a rabbit polyclonal antiserum to human placental aromatase (US Biological). Aromatase activity was assayed measuring the E_2 concentration (Immulite 2000, Siemens) in the cell culture medium after the addition of testosterone (100 nM) as an aromatase substrate, for 6 h. Each sample was analyzed in duplicate and at least three times.

Human Steroidogenic Tissues and Human Cell Lines

Human placental tissues (n = 2) were obtained from normal term deliveries, one of them from a male newborn (38 weeks) and the other from a female newborn (40 weeks), immediately after delivery in collaboration with the National Public Cord Blood Bank, Hospital Garrahan, Buenos Aires, Argentina. Exclusion criteria were maternal hypertension, diabetes, or a reduced amount of amniotic fluid at term. Human testicular tissues of patients (n = 3) aged 20 days, 2 months, and 3 months without any previous endocrine or metabolic diseases were obtained from necropsies, at less than 6 h postmortem. Human adrenal tissues (n = 2) were obtained from multiorgan transplantation donors (aged 14 and 21 years), less than 24 h after the diagnosis of brain death.

BeWo and H295R cells were purchased from the American Tissue Culture Center (Manassas, Va., USA). A Leydig cell tumor was obtained from a 1-year-old patient. Its microscopy was characterized by large polygonal cells with marked variations in shape and size, arranged in clusters separated by fibrovascular stroma. The tumor presented extensive areas of calcification. The cells had abundant eosinophilic cytoplasms, and many of them contained lipofuscin pigment. Reinke's crystals were observed. Clinical (phenotype) signs and a primary culture of the Leydig cell tumor confirmed a high production of testosterone.

cDNA samples of each type of steroidogenic human tissue were pooled before PCR. The study was approved by the Ethical Review Board of the Garrahan Pediatric Hospital, Buenos Aires, Argentina.

Results

Genetic Analysis

Genomic DNA analysis of the cP450arom gene *(CYP19A1)* of the patient, parents, and 3 siblings (2 boys and 1 girl) showed an intronic homozygous mutation (IVS9+5G>A) in the patient and heterozygosity for the same mutation in the parents and siblings. As the mutation is localized five bases from the 5′ beginning of intron 9, we suspected that normal splicing might be impaired. By using several splicing prediction programs (Splice View, Maxent; NetGene2; Fruitfly; ESEfinder 3.0) [24–28], it was confirmed that the splicing donor site disappears in the presence of the mutation, resulting in retention of intron 9.

mRNA Aromatase Study in the Patient

In order to evaluate if the mutation is involved in disrupting aromatase normal splicing in the patient, RT-PCR analysis was performed on total RNA of the patient's PBL. Primers were chosen according to the expected splicing event. For normal splicing, primers binding on exon 8 and exon 10 were selected. If intron 9 is retained as a result of an aberrant splicing event, primers binding on exon 8 and intron 9 would reveal this event. Amplification of a 633-bp fragment was observed after PCR using exon 8 and exon 10 primers (E8–E10) in the normal control but not in the patient. Sequence analysis confirmed exon 8-exon 9-exon 10 normal splicing. PCR amplification using primers binding on exon 8 and intron 9 (E8–IN9) revealed a 358-bp fragment in the patient but not in the normal control. Sequence analysis confirmed exon 8-exon 9-intron 9 splicing and the presence of the IVS9+5G>A mutation (fig. 1a).

Splicing Assays

In order to confirm that the intron 9 abnormal splicing found in the patient was a consequence of the point mutation, a splicing assay was developed. Splicing assays were performed in Y1 cells transfected with WT or Mut minigene constructions as described in the Materials and Methods section. RT-PCR analysis of total RNA extracted from Y1 transfected cells using primers binding on exon 8 and exon 10 (E8–E10) revealed a 633-bp fragment in WT and a shorter fragment (391 bp) in Mut, corresponding to exon 8-exon 9-exon 10 normal splicing and exon 8-exon 10 abnormal splicing, respectively, confirmed by sequencing (fig. 1b). Amplification using primers binding on exon 8 and intron 9 (E8–IN9) revealed a 358-bp fragment in Mut. Sequence analysis confirmed exon 8-exon 9-intron 9 splicing and the presence of the IVS9+5G>A mutation (fig. 1c).

Protein Analysis

In order to analyze the protein expression and aromatase activity of the mutated transcript, we developed transfection assays in COS7 cells with an expression vector (pcDNA3) containing cP450arom cDNA including part of intron 9 and the mutation (p-AroIn9Mut); a complete WT P450arom cDNA expression vector (p-AroWT) was used as normal control. Western blot analysis of total proteins extracted from COS7 transfected cells revealed the expected band of 53 kDa with p-AroWT and a shorter band of apparent 46 kDa with p-AroIn9Mut (fig. 2). Aromatase activity was assayed measuring the E_2 concentration in the cell culture medium after the addition of

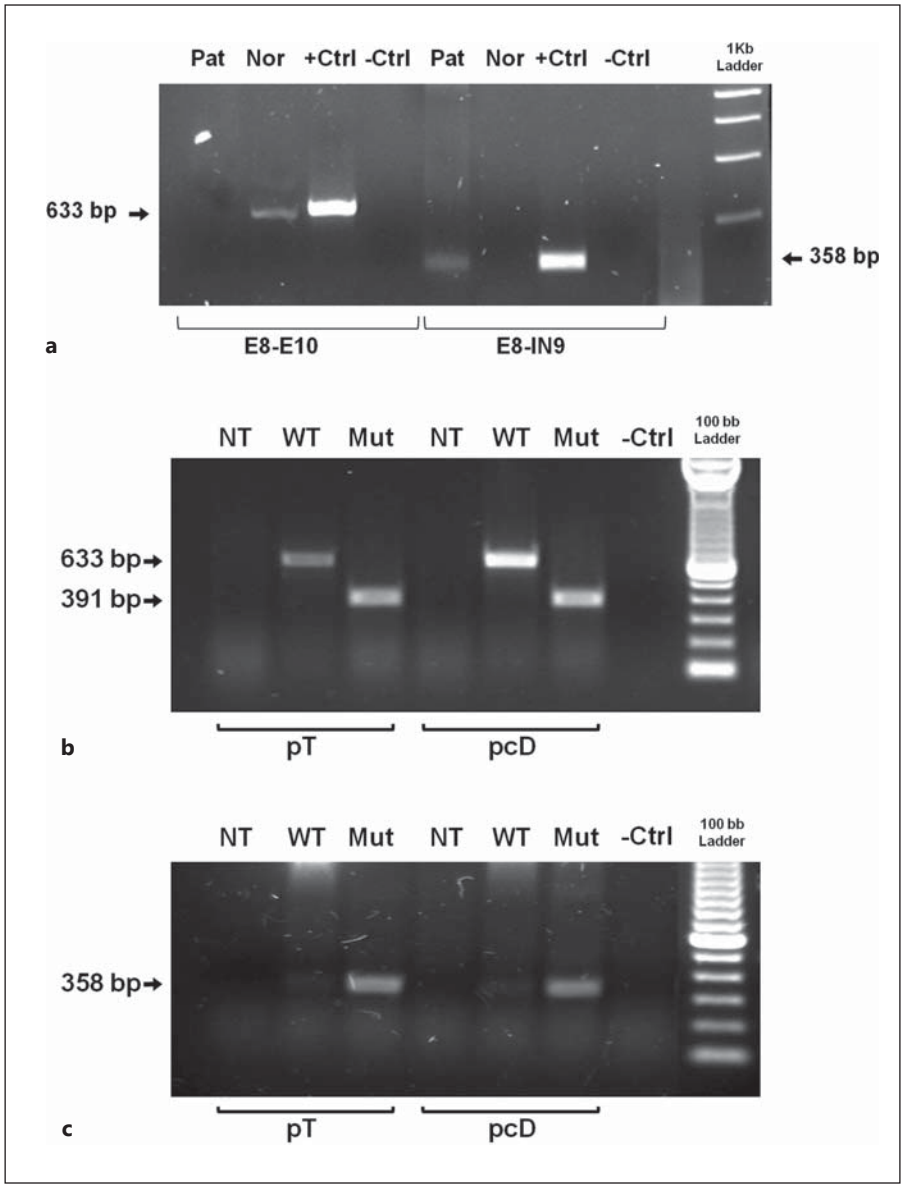
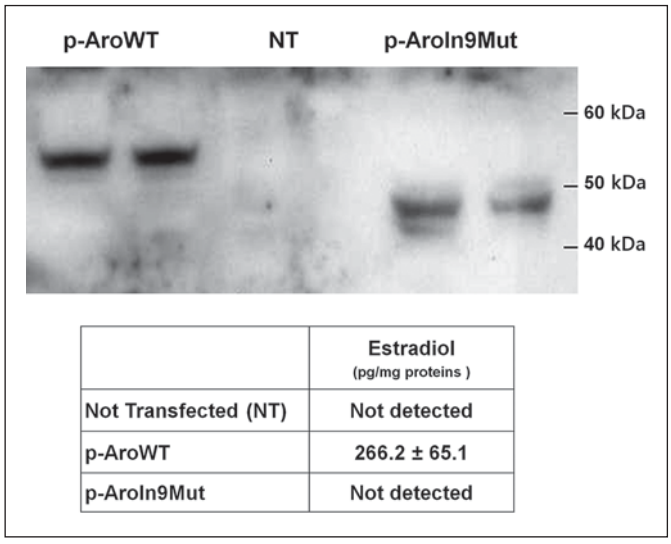


Fig. 1. a RT-PCR of total RNA from PBL of the patient (Pat) and a normal control (Nor) using exon 8 and 10 primers (E8–E10) or exon 8 and intron 9 primers (E8–IN9). cDNA from human placental tissues was used as a positive control (+Ctrl) and no input cDNA as a negative control (–Ctrl). **b**, **c** RT-PCR of total RNA extracted from Y1 cells transfected with WT or Mut minigene constructions using exon 8 and 10 primers (**b**) or exon 8 and intron 9 primers (**c**). NT = Not transfected; pT = pTarget vector; pcD = pcDNA3 vector.

testosterone as an aromatase substrate. The E_2 concentration was 266.2 ± 65.1 pg/mg protein in cells transfected with p-AroWT, but no E_2 was detected in nontransfected cells (controls) or in cells transfected with p-AroIn9Mut, even though the expression of a shorter protein was confirmed using Western blot.

Intron 9 Transcript Variant Is Present in Human Steroidogenic Tissues

In order to evaluate the presence of the P450arom-In9 mRNA variant in human tissues, an RT-PCR analysis of total RNA from the human placenta, adrenal glands, and testis was performed with specific primers. In the three normal steroidogenic tissues, PCR amplification revealed the expected aromatase 358-bp fragment containing a short sequence of unspliced intron 9 (confirmed by sequencing). The same analysis on total RNA from human tumor BeWo and H295R cell lines and Leydig cell tumor cells resulted in P450arom-In9 PCR fragment amplification (fig. 3a). Reverse-transcribed products were also amplified with specific primers binding on exon 9 and exon 10, revealing the expression of the full-length mRNA in all samples (fig. 3b). PCR amplification of β-actin as the housekeeping gene is shown in figure 3c.



	Estradiol (pg/mg proteins)
Not Transfected (NT)	Not detected
p-AroWT	266.2 ± 65.1
p-AroIn9Mut	Not detected

Fig. 2. Western blot analysis of aromatase protein expression in COS7 cells transfected with p-Aro, p-AroIn9Mut, or nontransfected. No P450arom protein was detected in nontransfected cells. Both the full-length aromatase (55 kDa) and a smaller 46-kDa protein probably associated with the In9 protein variant of aromatase were revealed.

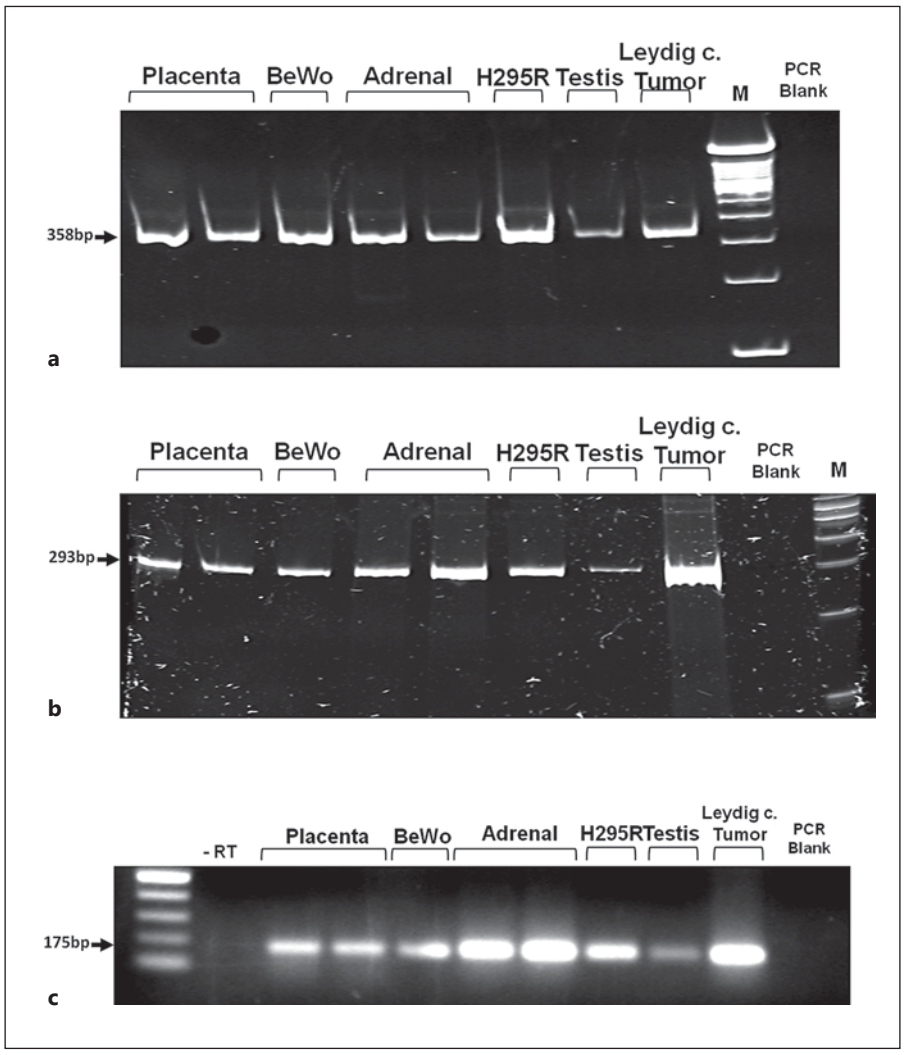


Fig. 3. Expression of the intron 9 mRNA variant in human tissues (placenta, adrenal gland, and testis), cell lines (BeWo and H295R), and Leydig cell tumor tissue (Leydig c. tumor). RT-PCR amplification products were separated and visualized on 10% polyacrylamide gel. **a** Amplification of the intron 9 variant, 358-bp product. **b** Amplification of the full-length variant, 293-bp product. **c** Amplification of β-actin, 175-bp product. M = 100-bp marker; PCR blank = no input cDNA; –RT = RT blank.

Discussion

We report a novel IVS9+5G>A mutation in the *CYP19A1* gene in a patient with an aromatase deficiency phenotype that generates an abnormal spliced mRNA that includes intron 9 in the patient's lymphocytes. Splicing assays using minigene constructs carrying the mutation confirmed that the IVS9+5G>A mutation impairs the normal splicing event.

The presence of an in-frame stop codon (TAA) 18 bp downstream of the splice junction in intron 9 would result in the synthesis of a truncated (46-kDa) and inactive aromatase protein lacking the heme-binding region found in exon 10. The transfection in COS7 cells of a cDNA composed of aromatase exon 2 to exon 9 plus the first 264 nucleotides of intron 9 carrying the patient mutation showed the expression of a shorter and inactive protein.

Up to the present, this is the fourth aromatase-deficient female described with a point mutation affecting normal splicing [23, 29, 30]. Unexpectedly, she presented pubertal E_2 levels at 13.5 years (27.7 pg/ml). As the patient mutation is placed close to the 5′ beginning of intron 9 and affects the normal splicing, the serum E_2 level might be explained by some normal splicing events that would be finally translated to an active aromatase. Similar findings were described by Shozu et al. [4] in a female with a homozygote splicing mutation (IVS6 ds +2 T–C); they found normal E_2 levels in the infant's serum and speculated whether the aromatization defect existed only in the placenta. In line with this, the follow-up of the aromatase-deficient patient described by Belgorosky et al. [23] and later on by Guercio et al. [31], presenting a splicing alteration, showed a slight elevation of serum E_2 levels (73.8 pmol/l) at the age of 7.7 years. The molecular study revealed that the mutation was associated with the expression of an aromatase mRNA lacking exon 5 and that in normal human steroidogenic tissues two alternatively spliced transcripts were normally expressed: the complete and the –Ex5. Therefore, it was proposed that the transcription of some mRNA that would include exon 5 and could be translated to an active aromatase explains the partial phenotype in the patient [21].

Similar to the latter, in the present study, an alternative splicing event that would include intron 9 was detected in human steroidogenic tissues. The partial aromatase activity found in the affected girl suggests that the mutation may disrupt the physiological mechanism involved in the regulation of alternative splicing of intron 9.

As previously described by Guercio et al. [31], two different estrogen replacement treatments were ineffective in suppressing gonadotropin levels, particularly FSH levels. Even though in this aromatase-deficient patient serum inhibin B levels were not measured, it is unlikely that the lack of suppression of the serum FSH level might be related to a low level of serum inhibin B. Accordingly, in a recent aromatase-deficient patient, reported by Burckhardt et al. [32], the upper normal serum inhibin B levels detected were unable to suppress serum FSH levels under low E_2 dose treatment. In addition, a high dose of estrogen replacement therapy was required to suppress serum FSH. Therefore, it might be speculated that this could be favored by the fact that patients have a lower aromatase activity, not only at ovarian levels, but also at local hypothalamic-pituitary levels.

Finally, the molecular studies explain the clinical phenotype of aromatase deficiency found in the affected girl. Moreover, hormonal studies confirm the role of estrogen in the feedback mechanism of gonadotropin secretion in pubertal females.

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