Molecular prenatal exclusion of familial partial androgen insensitivity (Reifenstein syndrome)

Serge Lumbroso¹, Jean-Marc Lobaccaro³, Charles Belon¹,*, Serge Amram², Bruno Bachelard³, Patrick Garandeau⁴ and Charles Sultan¹,⁴

Unité de Biochimie Endocrinienne du Développement et de la Reproduction¹, Hôpital Lapecronique, et Institut National de la Santé et de la Recherche Médicale INSERM US8, Montpellier, France; Service de Pédiatrie², Hôpital Carrémau, Nîmes, France; Service de Gynécologie-Obstétrique¹, Hôpital A. de Villeneuve, Montpellier, France; Endocrinologie et Gynécologie Pédiatiques³, Service de Pédiatrie I, Hôpital A. de Villeneuve, Montpellier, France


In a large family with Reifenstein syndrome, we previously performed molecular analysis of the androgen receptor gene. Direct sequencing showed a G→A point mutation at position 2818 of exon 7, which was responsible for an arginine–histidine substitution at position 840 of the androgen receptor. In this family, the proband's mother became pregnant and wished to know whether she was carrying an unaffected fetus. Polymerase chain reactions of the sex-determining region of the Y chromosome (the SRY gene) on trophoblastic DNA at week 14 revealed a 46,XY genotype. Sequencing analysis showed the canonical sequence (CGT, encoding an Arg residue), suggesting that the fetus was not affected. The expectation of normal male sexual development was confirmed by detection of normal male external genitalia through ultrasonography at week 24. These data confirm that sequence analysis of the androgen receptor gene on trophoblastic DNA is the most reliable method for prenatally diagnosing or excluding androgen insensitivity syndrome in high-risk families.

Charles Sultan, Endocrinologie et Gynécologie Pédiatriques, Service de Pédiatrie I, Hôpital A. de Villeneuve, 34059 Montpellier Cedex, France

Defects in the androgen receptor (AR) lead to androgen insensitivity syndromes, which constitute 50–70% of male pseudohermaphroditisms (1). These syndromes involve a variety of clinical disorders. A female phenotype in a 46,XY person constitutes complete androgen insensitivity syndrome. Partial androgen insensitivity (PAIS) is more complex and ranges from men with important ambiguous genitalia to men with infertility and/or minor degrees of undervirilization (2).

The familial form of PAIS is called Reifenstein syndrome (3). Children affected with this syndrome present with micropenis, small testes and various genital abnormalities, i.e. hypospadias, bifid scrotum and vaginal-pouch remnants (3). The severity of such ambiguous genitalia often leads to an initial assignment to the female sex or to a delay in sex assignment, even though the patients are reared as males in most cases. This creates a stressful situation for parents. Furthermore, at puberty the patients develop gynaecomastia and present poor virilization, causing considerable psychological distress in adult life. Consequently, there is a high demand for prenatal diagnosis in families already afflicted with this syndrome. In our past experience, before genetic counselling and prenatal diagnosis were allowed by molecular genetics, parents often requested therapeutic abortion when the fetus was caryotypically male.

Male sexual differentiation depends on the action of testosterone and dihydrotestosterone during fetal life, childhood and puberty (2). These hormones act via the AR. The AR gene has been located at position q11-12 on the X chromosome (4) and belongs to the nuclear receptor family with common structural organization in three domains (5). The amino-terminal domain, encoded by exon 1 of the AR gene, participates in regulation of transcriptional activity. The central DNA-binding domain, the most conserved among family members, includes two zinc-finger structures encoded by exons 2 and 3, respectively. The carboxy-terminal domain is the site of androgen binding to the receptor and is encoded by exons 4–8. Cloning and sequencing (6) of the AR gene have provided the molecular tools required to perform prenatal diagnosis, either through identification of the causative mutation (7, 8) or polymorphism of the AR gene (9, 10).

In a large family with three brothers presenting with ambiguous genitalia, hormonal data along with AR biochemical and molecular analyses led to the diagnosis of Reifenstein syndrome. The patient's sister already had a child affected with the same disease and requested a prenatal diagnosis in a second pregnancy.

* Permanent address: Laboratoire de Biochimie, Faculté de Pharmacie, Montpellier, France.
Fig. 1. Genealogy of the studied family with partial androgen insensitivity syndrome: (■) affected subjects, (○) obligate carriers. The arrow indicates the fetus.

Subjects and methods

Family

Figure 1 shows the family genealogy. Subjects II-1, II-2 and II-3 presented the same severe ambiguous genitalia (Fig. 2). The main clinical and hormonal data are summarized in Table 1. Male sex of rearing was assigned at birth.

Subject III-1 had the same ambiguous genitalia as his three maternal uncles, i.e. severe perineal hypospadias, micropenis, bifid scrotum and cryptorchidism (Fig. 3). At 7 weeks, the plasma testosterone level was raised from 34 to 54 nmol/l under hCG stimulation (7 x 1500 U). Plasma FSH and LH levels were, respectively, 2.4 and 3.7 IU/l (FSH: 0.16 < N < 4.1 IU/l; LH: 0.02 < N < 7 IU/l).

Determination of androgen-binding capacity

An androgen binding study was performed on cultured genital skin fibroblasts (11) of the four affected subjects. The number of sites and dissociation constant of the AR were determined by Scatchard analysis using 17-methyl[3H]-R1881 binding (methyltrienolone; Dupont de Nemours, France).

Deoxyribonucleic acid extraction

Genomic DNA of the four affected subjects as well as the mother (II-4) were extracted from peripheral blood lymphocytes by phenol–chloroform extraction and ethanol precipitation (12). The trophoblast was biopsied at 14 weeks' gestation and DNA was extracted according to Lobaccaro et al. (9).

Fig. 2. External genitalia of subjects II-1, II-2, II-3 and III-1 at the age of endocrine investigation, i.e. 154/12, 135/12, 106/12 and newborn respectively.
Table 1. Clinical data and endocrine profile of subjects II-1, II-2 and II-3.

<table>
<thead>
<tr>
<th></th>
<th>II-1</th>
<th>II-2</th>
<th>II-3</th>
</tr>
</thead>
<tbody>
<tr>
<td>Chronological age</td>
<td>15/12</td>
<td>13/12</td>
<td>16/12</td>
</tr>
<tr>
<td>Bone age</td>
<td>12/12</td>
<td>10</td>
<td>9</td>
</tr>
<tr>
<td>External genitalia</td>
<td>Perineal hypospadias</td>
<td>Perineal hypospadias</td>
<td>Perineal hypospadias</td>
</tr>
<tr>
<td>Testes</td>
<td>Micropenis</td>
<td>Micropenis</td>
<td>Micropenis</td>
</tr>
<tr>
<td>Gynaecomastia</td>
<td>Yes</td>
<td>No</td>
<td>No</td>
</tr>
<tr>
<td>Pubic hair</td>
<td>PO</td>
<td>P0</td>
<td>P0</td>
</tr>
<tr>
<td>Height</td>
<td>-3 SD</td>
<td>-2 SD</td>
<td>0 SD</td>
</tr>
<tr>
<td>Testosterone (nmol/l)</td>
<td>22.54</td>
<td>2.43</td>
<td>&lt;0.35</td>
</tr>
<tr>
<td>DHT (nmol/l)</td>
<td>1.73</td>
<td>1.08</td>
<td>&lt;0.10</td>
</tr>
<tr>
<td>E2 (pmol/l)</td>
<td>1.52 ± 0.70</td>
<td>0.35 ± 0.24</td>
<td>0.45 ± 0.35</td>
</tr>
<tr>
<td>FSH (IU/l)</td>
<td>85 ± 47</td>
<td>37 ± 18</td>
<td>37 ± 11</td>
</tr>
<tr>
<td>LH (IU/l)</td>
<td>8.1 ± 3.2</td>
<td>3.8 ± 1.8</td>
<td>2.2 ± 1.1</td>
</tr>
</tbody>
</table>
<br />
*Ranges of values according to the age of the patients are indicated by N. DHT: dehydrotestosterone; E2: oestradiol-17β; FSH: follicle-stimulating hormone; LH: luteinizing hormone.*

**Enzymatic amplification**

Polymerase chain reactions of exons 2–8 of the AR gene were performed in a 50-µl final volume using 500 ng of genomic DNA, 150 ng of each primer, 200 µmol/l of each nucleotide, 1.5 mmol/l MgCl2, 10 mmol/l TRIS-HCl (pH 8.3), 50 mmol/l KCl, 0.01% gelatine and 0.5 U of Taq polymerase. Reactions were cycled for 0.5 min at 95°C, 0.5 min at 58°C and 0.5 min at 72°C for 35 cycles. Polymerase chain reaction of the SRY sex-determining gene was performed under the same conditions but with an annealing temperature of 65°C (13). Amplified samples were submitted to electrophoresis on a 1.2% agarose gel stained with ethidium bromide to verify the product sizes.

**Direct sequencing**

Polymerase chain reaction (PCR) products of exon 7 were purified on 2% Nusieve gel (FMC, USA) followed by phenol–chloroform extraction. 0.25 pmol of the

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*Fig. 3. Partial sequence of androgen receptor exon 7 showing the G to A substitution that changed the sense of codon 840 from Arg to His in the proband. The G to A transversion is marked with an asterisk. The mother's sequence showed the mutant and normal alleles (A/G) and is marked with a double asterisk. Two control sequences are shown: the father's sequence and a 46,XY normal male (indicated by C). The sequence of the fetus was normal.*
Table 2. Biochemical characteristics of the androgen receptor (AR) determined on genital skin fibroblasts of the four affected subjects.

<table>
<thead>
<tr>
<th>Patient</th>
<th>II-1</th>
<th>II-2</th>
<th>II-3</th>
<th>III-1</th>
</tr>
</thead>
<tbody>
<tr>
<td>$B_{\text{max}}$</td>
<td>235</td>
<td>110</td>
<td>96</td>
<td>&lt; 80</td>
</tr>
<tr>
<td>$K_d$</td>
<td>0.33</td>
<td>0.2</td>
<td>0.3</td>
<td>ND</td>
</tr>
</tbody>
</table>

*The androgen-binding activity ($B_{\text{max}}$) and the dissociation constant of the androgen-AR complex ($K_d$) of the patients are the mean of two values. Normal values are $650 \pm 200$ nmol/kg DNA for $B_{\text{max}}$ and $0.6 \pm 0.3$ nmol/l for $K_d$. The normal ranges for the assays are expressed as means $\pm$ 2sd.

template was sequenced with 10 pmol of primers L3 (5' CATCCACATCGTCCAGTGG 3') or L5 (5' ATCGTTGTGTTACAGGCTGCA 3'), as described by Casanova et al. (14). Sequencing reactions were repeated twice with two different PCR products.

Results

The clinical presentation and endocrine data for affected members of the family along with the X-linked inheritance of the disease, as pointed out by the genealogy (Fig. 1), suggested that ambiguous genitalia was due to PAIS. This was confirmed by the receptor binding assay, which showed a severe decrease in the receptor binding activity (Table 2).

The normal fragment lengths determined on agarose gel after electrophoresis of the PCR products of the affected subjects demonstrated the absence of gross deletions in exons 2–8. Moreover, the reduced androgen-binding sites suggested the presence of an alteration within the androgen-binding domain of the AR encoded by exons 4–8. We found previously a G–A transversion at position 2088 in exon 7, which changes the sense of codon 840 from Arg to His (15) (numbering is as defined by Lubahn et al. (6)). This point mutation was found in the four affected members of the family.

For the prenatal sex diagnosis, PCR on the trophoblastic DNA of the SRY gene, which is known to be the testis determining factor (16), showed a fragment of the expected size of 648 bp, indicating within 48 h that the genotypic sex of the fetus was male. Subsequently we sequenced the suspected exon of the AR gene of the trophoblast DNA and found the canonical sequence (Fig. 4). We thus considered that the fetus was not affected.

Ultrasoundography at 20 weeks confirmed normal male development of the external genitalia of the fetus.

Discussion

Prenatal diagnosis of PAIS is a difficult area. Newborn infants presenting with ambiguous genitalia create an immediate problem in diagnosis and management because of the delayed or even inappropriate gender assignment, in addition to creating a stressful situation for parents. Indeed, if genital abnormalities are severe, complex corrective surgical procedures have to be performed, without any guarantee of acceptable masculinization of external anatomy and efficacious functioning. Undoubtedly this can cause considerable psychological distress in adult life. Thus, in high-risk families, prenatal diagnosis may be an option for subsequent pregnancies.

The high variability of androgen gene alterations in androgen insensitivity syndrome makes antenatal diagnosis a difficult procedure. Indeed, PCR or restriction fragment length polymorphism-detected gross deletions, which would permit easy diagnosis, are very rare (8, 17). Point mutations are thus the most common cause of androgen insensitivity syndrome. However, there are many and they are located all along the AR gene without any significant helpful hot spots, even though two clusters of AR gene mutations have been described in the ligand-binding domain (18). Identification of mutations associated with androgen insensitivity syndrome in a family is thus long and
tedious work that requires sequencing of the 8 exons. A prior screening step is useful, using either single-strand conformation polymorphism (12, 19) or denaturing gradient gel electrophoresis (20). However, these screening methods are not 100% efficient. In some cases, genetic abnormalities responsible for the disease have yet to be detected.

In such situations, AR gene polymorphisms must be studied. Two polymorphisms have been described so far. Hind III polymorphism (4) is not very informative because it only concerns 10% of the X chromosomes. However, it allowed us to obtain prenatal diagnosis of PAIS (10) in one family and carrier diagnosis in another (21). The second is the CAG polymorphism in exon 1 of the AR gene (22). This polymorphism is much more informative because heterozygosity occurs in 50–85% of cases (23–25). We reported recently two prenatal exclusion diagnoses of PAIS using CAG polymorphism (9). However, these polymorphisms are only an indirect demonstration and genetic recombinations of maternal mutant and wild alleles cannot be excluded, even though the risk is very low because of the intragenic location of markers. Once the mutation is detected, its causal role in the development of the disease must be proved by in vitro studies of the mutated AR obtained by direct site mutagenesis. These experiments have not been performed yet on the Arg-His 840 substitution, but its association with androgen insensitivity syndrome in different unrelated subjects (18, 26) is a sufficient argument for its causal role. Under these conditions, the absence of this Arg-His 840 mutation within the trophoblastic DNA indicates that the fetus is not affected.

McPhaul et al. (18) and Pinsky et al. (26) described the Arg-His 840 substitution in four unrelated families with androgen insensitivity syndrome. Association of the same Arg-His 840 substitution with different androgen insensitivity phenotypes and different biochemical characteristics of the androgen receptor (18, 26) have pointed out the complexity of the genotype–phenotype relationship in androgen insensitivity.

Although carrier status in Reifenstein syndrome can be determined when the causative mutation has been identified, the severity of ambiguous genitalia in an affected offspring cannot be predicted reliably with certainty. Indeed, in rare families with PAIS considerable phenotypic variability has been reported (27, 28). The reason for such phenotypic variation remains unclear but it is probably dependent on more than AR gene abnormalities alone. In the family studied here, the three maternal uncles and the affected child had the same frankly ambiguous external genitalia. This indicates that the same mutation was responsible for the same undervirilization in the family.

In conclusion, detection of the causative mutation (or use of AR gene polymorphisms) is a critical tool for prenatal diagnosis of androgen insensitivity syndrome in high-risk families.

References

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Received September 7th, 1993
Accepted November 1st, 1993

Note added in proof: Since acceptance of this paper, the mother has given birth to a boy with normal male external genitalia.