

Androgen insensitivity syndrome (or Morris syndrome) and other associated pathologies

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Abstract. – Background: The androgen insensitivity syndrome (AIS) is a disease connected with the inactivation of AR due to a mutation that inactivate male sexual differentiation, and causes a spectrum of phenotypic anomalies having as a common aspect the loss of reproductive characteristics.

Patients: In this paper the clinical, endocrinological and molecular features of three patients are reported. The first case concerns a 17 years old patient, the second an infant suffering from inguinal hernia that has been surgically corrected when he was 3 months old and finally the third case concerns a 57 years old woman.

Results: All the subjects had a common primary amenorrhea and a following pelvic echography highlighted the absence of internal genital organs and the presence of a dead-end vagina. All the patients are characterized by a normal male karyotype and present on the short arm of the Y chromosome the SRY gene. Moreover, FISH revealed the presence of androgen receptor gene on the X chromosome and the SRY gene on the Y one.

The automatic sequencing of the genes for the androgen receptor (AR) shows that each subject has a mutation in the gene for the androgen receptor (AR). These mutations are associated with complete androgen insensitivity syndrome (CAIS). All the cases of study exhibited a negative family history for CAIS.

Conclusion: This study confirms the need to perform a pelvic examination by means of echography in pre-pubertal and pubertal age to highlight the normality of the internal genitalia (uterus and ovaries). If a primary amenorrhea is checked, the karyotyping is compulsory.

If a Morris's syndrome is suspected, it is of critical importance to find the rudimentary male go-

nads (by means of MRI, pelvic echography, laparoscopy) and surgically remove them to prevent the onset of malignancies (teratoblastoma, gonadoblastoma). Once the considered disease has been identified, a continuous psychological help can be considered useful for the patient and the family.

Key Words:

Androgen insensitivity syndrome (AIS), Androgen receptor (AR), XY female.

Introduction

The process of sex characterization and its differentiation in male and female can be considered as the result of many events. Each one is fixed by the previous and produces the following one. Particularly, four main steps can be identified:

- Genetic sex detection. It depends on gonosomes assortment (XX for the female case and XY for the male one);
- Gonadal sex determination (ovary/testicle);
- Phenotypical sex differentiation (characteristics internal and external genitals);
- Gender identity acquisition (female or male).

The first event is a random process that is due to the male fertilizing gamete, and depends on the presence inside its haploid chromosome set of a X or Y chromosome. The last event is real-

ly complex, not well known from the regulating mechanisms point of view and strongly influenced by environmental factors. Defects in androgen action may compromise sexual development in genetically male individuals, this resulting in an array of phenotypes, commonly known as androgen insensitivity syndrome (AIS)¹. This paper presents the clinical, endocrinological and molecular features of three patients with normal male karyotype (46,XY) and complete phenotype reversion from male to female (complete androgen insensitivity syndrome = C.A.I.S.)².

Physiopathological Mechanism

In a 46,XY karyotype fetus the primitive gonad differentiation into testicle depends on a gene located on the short arm of chromosome Y, called "S.R.Y." (Sex Determining Region on the Y chromosome)³⁻⁵. The regular development of internal and external genitals towards male features depends on the hormonal secretion of the fetus testicle, that – starting from the second pregnancy month – produces two hormones: testosterone and the anti-müllerian hormone^{6,7}. The latter is a glycoprotein produced by Sertoli's cells that acts through the inhibition of the Müller structures, that produces uterus and fetus female annexes⁸⁻¹⁰ (Figure 1).

Testosterone, produced by Leydig's cells, is the main steroidal hormone with androgenic action produced by the testicle and the most present in the body. When this hormone through a passive diffusion process penetrated into target cells, it acts its hormonal function as well as is partially metabolized in the other most important androgen, the 5 α -dihydrotestosterone, by the enzyme 5 α reductase.

During the embryogenesis the testosterone directly acts on wolffian ducts inducing their differentiation into internal male genitals (vasa deferentia, epididymis, seminal vesicles), while the 5 α -dihydrotestosterone induces the differentiation of the genital tubercle and the labioscrotal folds in the male external genitals. To develop their effects, the testosterone and the 5- α dihydrotestosterone link at peripheral level with only one intracellular protein: the androgenic receptor (AR).

The interaction between receptor and hormone causes a topological modification in the structure of the receptor that becomes able to link itself with its specific acceptor on the genome (androgen-response element: ARE) with dimeric form. This leads to a subsequential activation of the proteic synthesis in the androgen dependent genes (Figure 2). In absence of the receptor or in presence of a functionally irregular receptor, androgens cannot develop their effects.

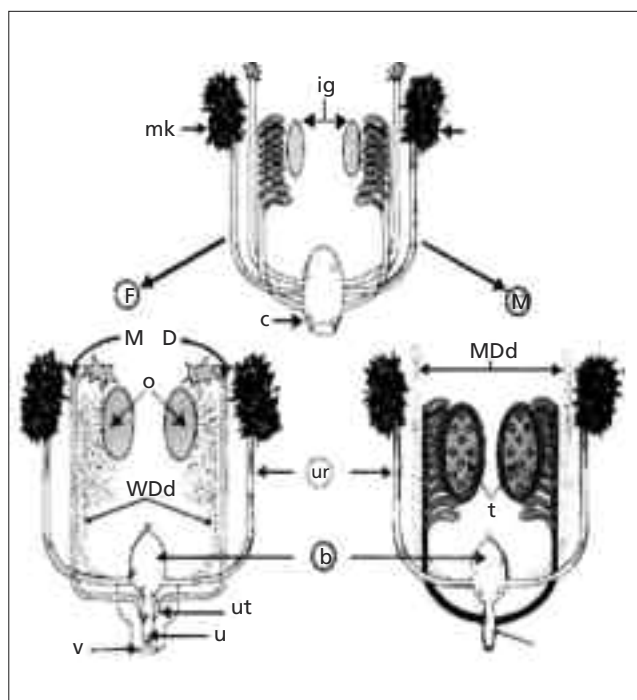


Figure 1. The presence of the SRY gene allows the formation of the male gonads, the presence of inhibitor Mullerian (MIF) determines the disappearance of Mullerian structure (uterus, fallopian tubes). ig = undifferentiated gonads; mk = kidney metanephros; M = Male; F = Female; ur = ureters; MD = ductus Muller; o = ovaries; WDD = Wolffian ductus degenerate; ut = uterus; u = urethra; MDd = Muller ductus degenerate; b = bladder; c = cloaca; t: testicle.

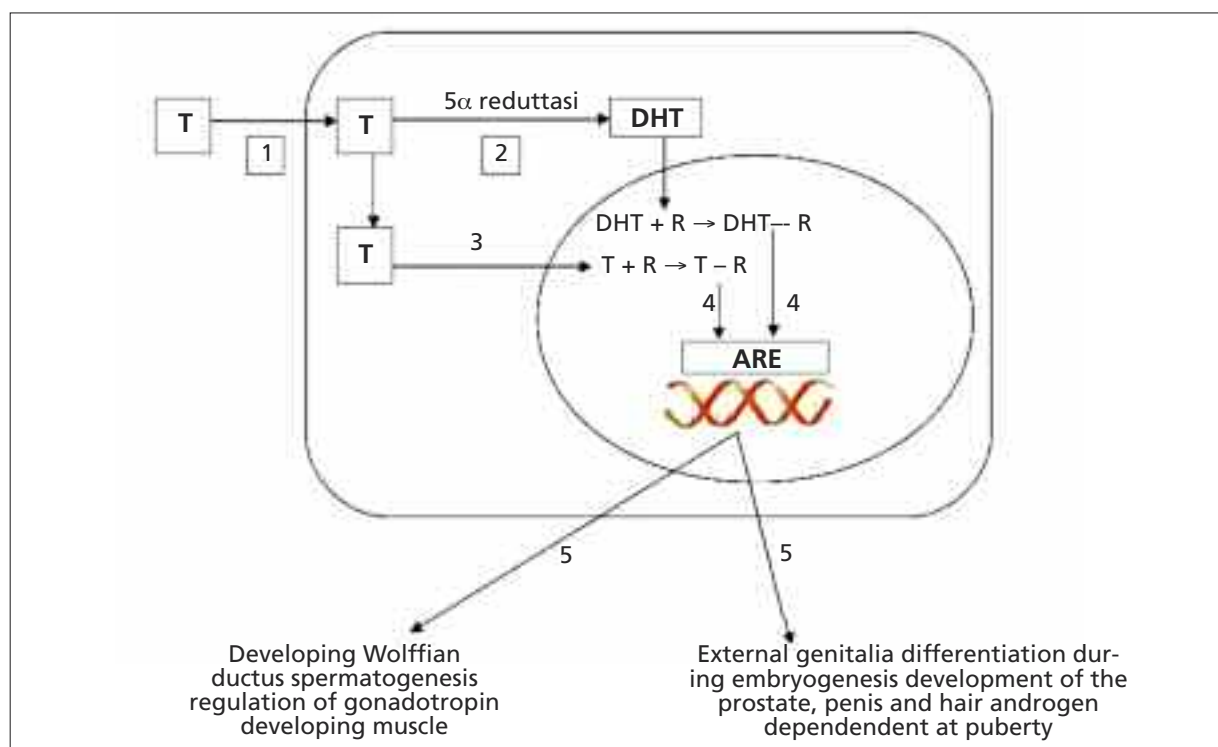


Figure 2. Are = androgen response element; T = testosterone; R = androgenic receptor; DHT = 5-alpha dihydrotestosterone.

As a consequence, this causes an incomplete partial androgen insensitivity syndrome (PAIS), or absent male differentiation of the fetus (CAIS). At puberty age this results in the absence of virilization phenomena even though there is the presence of normal male karyotype (46,XY)^{11,12}. Subjects characterized by complete androgen insensitivity syndrome (CAIS) show female external genitalia, usually with small labial folds, a short blind ending vagina, absent wolffian duct derived structures and prostate, gynecomastia, scanty pubic and axillary hair. In partial androgen insensitivity syndrome (PAIS), several different phenotypes are evident, with predominantly female phenotype (female external genitalia, pubic hair with or without litoromegaly and partially to completely fused labia) in most severe form, ambiguous genitalia to predominantly male phenotype with micropenis, perineal hypospadias and cryptorchidism in less severe forms. The latter group of patients is also named as Reifenstein's syndrome. To the PAIS affected patients a level is assigned according to the severity of androgen insensitivity and affinity of the phenotype with male or female pattern. Subjects with mildest form of androgen

insensitivity (MAIS) usually have normal male genitals and internal male structures, and during the puberty age they may have breast enlargement, sparse facial and body hair, and small penis. Some affected males may also have impaired sperm production resulting in oligozoospermia or azoospermia.

The Androgen Receptor (AR)

To perform their biological activity, testosterone as well as 5α-dihydrotestosterone¹³⁻¹⁵, have to link to their own specific receptor, the androgenic receptor (AR), which mediates their biological activity. About this aspect it is necessary to highlight that the 5α-dihydrotestosterone shows a greater affinity for AR with the respect to the testosterone. This critical receptor for male development is encoded by the *AR* gene mapped onto the long arm (Xq11-12) of the X-chromosome^{16,17} (Figure 3).

The androgenic receptor is a member of the superfamily of intracellular receptors for steroidal hormones and is a transactivating factor, i.e. it interacts with the target genes and triggers the transcription¹⁸. The gene has a length of about 90kb and includes 8 exons labeled with let-

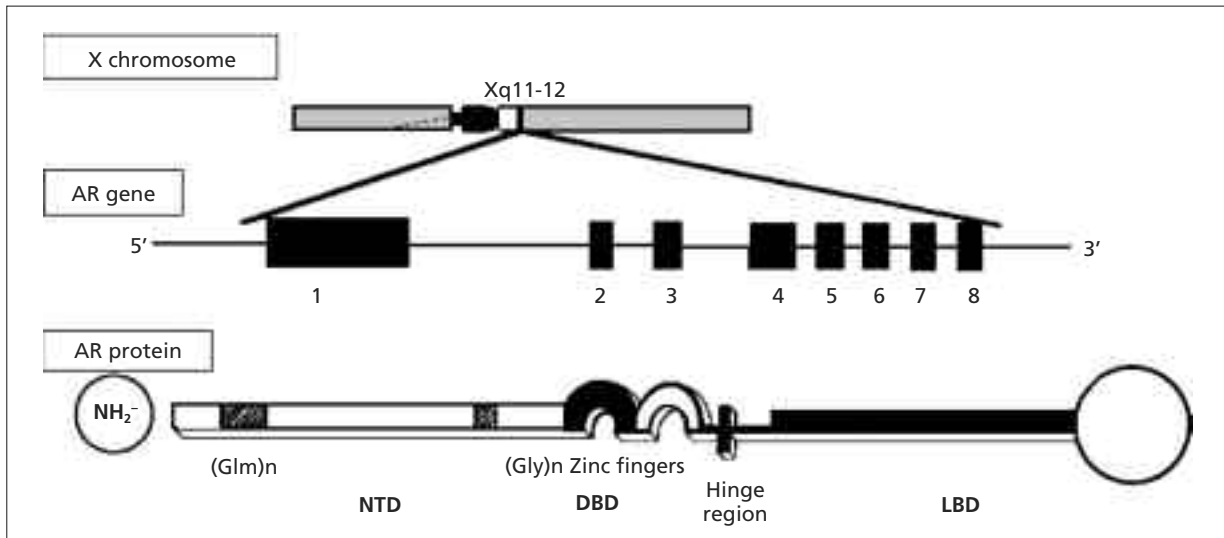


Figure 3. Human androgen receptor gene is mapped to the long arm of the chromosome X (Xq11-12). The human androgen receptor protein is encoded by 8 exons (1-8). Similarly to other nuclear receptors, the protein consists of several distinct functional domains: the N-terminal domain (NTD) containing two polymorphic stretches -(Gln)_n and (Gly)_n-, the DNA-binding domain (DBD), the Hinge region and the ligand binding domain (LBD).

ters A-H or numbers 1-8. The exons are separated by 7 introns. This gene codifies the AR receptor protein that is composed by 919 amino acids with a molecular weight of 110 Kd. The protein is characterized by four functional regions:

- N-terminal transactivation domain (TAD): long region NH₂-terminal (exon 1);
- DNA-Binding Domain (DBD): region that interacts with the target genes' DNA (exon 2 and 3);
- Hinge region (exon 4);
- Ligand Binding Domain (LBD): region that represents the link region for the steroid (exon 4-8).

This molecular structure is a common feature for all the members of the steroidal receptor superfamily. The NH₂ terminal region represents more than half of the receptor protein and contains the structures that regulate the transcription. Within the superfamily is the less conserved receptor part, since it can change in length because of a variable number of triplets CAG that codify the glutamine. The CAG forms a polyglutamine chain (PolyQ: variable number of repeated triplets between 21±2). In the normal subject the number of triplets is between 11 and 35. An increasing of the number is connected to the Kennedy's disease that is characterized by a progressive atrophy of spinal and bulbar muscles

and resistance to androgens¹⁹. In some cases, this is also related to male infertility²⁰. The DBD region is highly conserved. It contains two structures called zinc finger composed by four cysteines that together link a zinc atom.

The first zinc-finger, codified by the exon 2, is involved in the interaction between the receptor protein-DNA since it recognizes and links the responsive element (A.R.E.=Androgen Responsive Element). The ARE is a specific sequence of bases localized on the receptor target gene. The Hinge region is codified by a part of the exon 4 and is involved in the migration process of the receptor protein into the cellular nucleus. The region COOH-terminal, is codified by the remaining part of the exon 4 and by the exons 5-8. This region has the main task to link the hormone, this resulting into receptor activation. In the inactivity phase, when the receptor inside the cellular cytoplasm is not linked to the androgen, its COOH region is linked to some proteins known as heat shock proteins (hsp) (Figure 4)²¹. In this condition, AR is not able to migrate into the nucleus as well as to link to the target genes DNA. The interaction with the androgen leads to the dissociation between AR and hsp, the further phosphorylation and the migration into the cellular nucleus, where the receptor undergoes a process of homodimerization. This process is characterized by the each other linkage of the complex hormone-receptors, allowing the recognition and the link to

the responsive element present on the target gene and generating the synthesis of new specific proteins (Figure 4).

Cases Under Study

This work has the objective to characterize in the most accurate possible way, three clinical cases of Morris Syndrome²².

The first case refers to a 17 years old patient, female from the phenotypic point of view, with height of 175 cm and a weight of 58 kg. The patient approached the clinic with primary complain of absence of menarche. A detailed history of the patient was taken along with physical examination. Physical examination revealed poorly developed breasts, female external genitals, blind ending vagina and no pubic or axillary hair (Figure 5). Pelvic echography pointed out no development of Wolffian duct derived structures, well distended urinary bladder; in the inguinal region it was observed on both sides the presence of swellings which were identified as testes through the ecography. After their magnetic resonance imaging (M.R.I.), pelvic detection, surgical asportation and histologic test, pelvic echography result was confirmed (Figure 6).

The second case refers to a newborn with inguinal hernia diagnosis treated with surgery dur-

ing the third month of life. Once 13 years old, the patient was tall, thin and with no signs of puberty. The patient was also suffering from a mild-minor osteoporosis. Moreover, a pelvic echography highlighted the absence of uterus and ovaries. The uterus and ovaries absence was conformed with. pelvic M.R.I and Morris syndrome was suspected. The laparoscopy was performed pointing out the absence of the uterus, ovaries and fallopian tubes confirming that the patient was suffering from Morris syndrome.

The third case is referred to a 57 years old woman that was under investigation after a "amenorrhoea primaria" has beendiagnosed. Pelvic echography highlighted the absence of uterus and ovaries. The results coming from the pelvic echography were confirmed by the magnetic resonance imaging (M.R.I.) – without contrast medium – of the lower abdomen. The latter examination has shown the following:

- Uterus and ovaries not displayed.
- A short blind ending vagina
- Inguinal hernia.
- Small reactive lymphonodes in the inguinal region.
- Female external genitals with normal morphological characteristics.

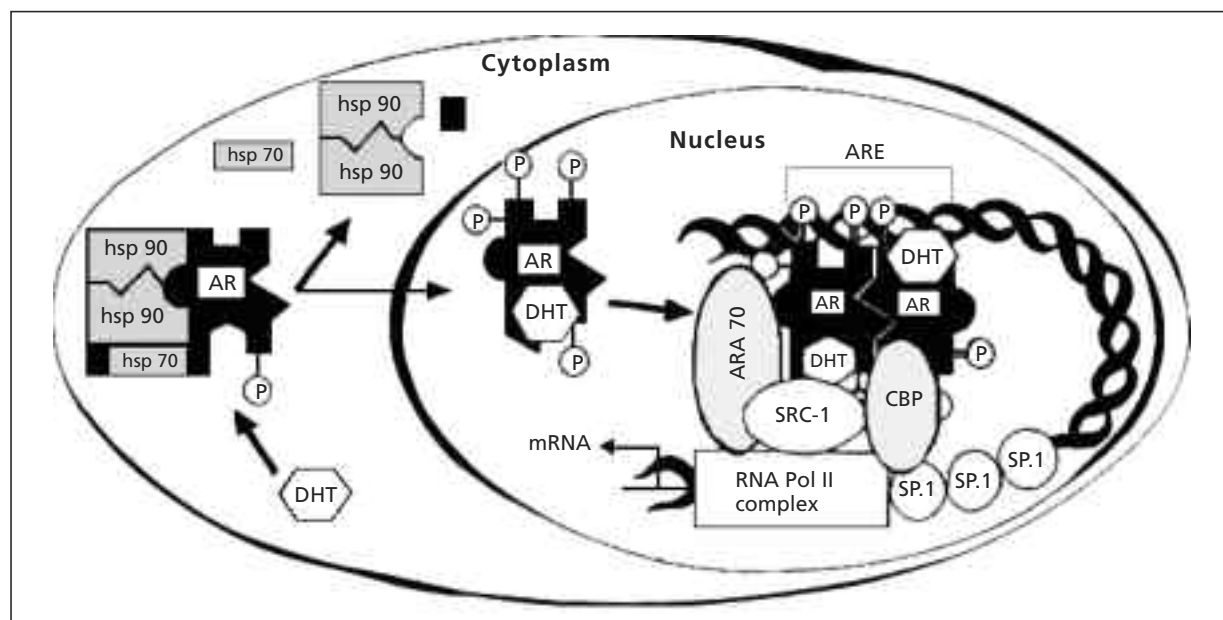


Figure 4. Ligand-dependent activation of the androgen receptor. Androgens such as DHT diffuse through the plasma membrane and bind to the AR. Upon ligand binding, the AR undergoes conformational changes involving an NH₂-carboxyl-terminal interaction and receptor stabilization. The AR translocates to the nucleus where dimerization and DNA binding to regulatory androgen response element occurs. AR = androgen receptor; DHT = dihydrotestosterone; CBP = CREB-binding protein; ARE = androgen response element; hsp = Heat shock protein; SRC-1 = steroid receptor coactivator 1).



Figure 5. Subject 17 years of phenotypically female, with clinical suspicion of Morris syndrome.

- Absent wolffian duct derived structures and prostate.
- Some biochemical exams have shown the following : testosterone 2.96 ng/ml (normal value male: 2.4-8.3/female: 0.14-0.8); follicle stimulating hormone (FSH) 8.7 mUI/ml (normal value: male 1.4-18.1); leutinizing hormone (LH): 28.5 mUI/ml (normal value: male 1.5-9.3), progesterone: 1.1 ng/ml (normal value: male: 0.28-1.22).

Material and Methods

In order to analyze the exact cause of the considered clinical cases, the following genetic analyses have been carried out:

- Analysis of the karyotype from peripheral blood: The karyotype has been obtained by T-lymphocytes extracted by peripheral blood us-

ing common cultural techniques. The chromosomes have been banded with GAG and CBG methods.

- Fluorescent in situ hybridization (F.I.S.H.). It's a hybridization method that allows, after the fixing of metaphases on a slide, identifying specific sequences in nucleic acids. This identification is done through probes marked with specific fluorophore that emit at different wavelengths. In this work the following probes have been used:
- LSI SRY/CEPX: is a DNA predenatured probe that hybridizes with the band Yp11.3 of Y chromosome. This is marked with the fluorophore spectrum orange. In the kit used this probe is mixed with another predenatured one that hybridizes with the centromere of the X chromosome (alpha satellite DNA of Xp11.1-11.1), marked with fluophore spectrum green. This allows highlighting with a single hybridization the position of SRY with the respect to the X chromosome.



Figure 6. Bilateral incision in the groin with extraction and subsequent removal of the gonads (case 1).

- LSI androgen receptor gene: is a predenatured probe for DNA of around 380 Kb that hybridizes with the region Xq11.2-Xq12 of the X chromosome that corresponds to the locus of the AR gene. Also in this case it is a predenatured probe marked with fluorophore spectrum orange.
- Molecular study focused on the determination of the presence/absence of the SRY gene [located on the short arm of the chromosome (Yp11.3)] and of specific STS (Sequences Tag Sites) located in the regions AZFa, AZFb and AZFc of the long arm of Y chromosome and studied for the screening of the microdeletions of Y chromosome. The selected STS agree the guidelines published in 2004 by the European Academy of Andrology (EAA) and by the European Molecular Genetics Quality Network (EMGQN). The following STS have been analyzed: 84 and 86 (AZFa), 127 and 134 (AZFb), 254 and 255 (AZFc). As a control, the gene ZFX/ZFY has been amplified. In order to do that, blood samples collected in EDTA-K3 have been used. The molecular analysis included the following steps:
 - DNA was extracted from peripheral blood (25 µl) using the kit of Promega Italia S.r.l. (DNA IQ™ System, cod.C6701).
 - Multiplex amplification of the sequences described above using a commercial kit (Experteam cod. AZ.020).
 - The product thus obtained is subjected to electrophoresis on agarose gel (3%) to 20', 30' to 70 mV.
 - Analysis of electrophoretic mobility was performed through UV transilluminator.
 - The result of amplification has been photographed and archived.
- The automatic sequencing of the gene for the androgen receptor (AR) develops by the following steps:
 - **Step 1, DNA purification.**
 - **Step 2, PCR Amplification e Cleanup.**
 - **Step 3, Cycle Sequencing:** is a easy method in which subsequent phases of denaturation, annealing, and extension in a thermal cycler result in linear amplification of extension products. The products are then injected into a capillary. All current Applied Biosystems DNA sequencing kits use cycle sequencing protocols.
 - **Step 4, Sequencing Reaction Cleanup:** After the sequencing reaction, it is important to remove unincorporated dye terminators

and salts that may compete for capillary electrophoretic injection. Unincorporated terminators can co-migrate with the sequencing template, this resulting in basecalling errors, and excess of salt leads to a poor signal-to-noise ratios.

- **Step 5, Capillary Electrophoresis:** After performing the post-sequencing reaction purification, samples are ready to be analyzed trough an Applied Biosystems capillary electrophoresis-based genetic analyzer. During capillary electrophoresis, the products of the cycle sequencing reaction migrate through capillaries filled with polymer. The negatively charged DNA fragments are size separated as they move through the polymer in the capillaries toward the positive electrode
- **Step 6, Data Analysis:** After electrophoresis, data collection software creates a sample file of the raw data. Using downstream software applications, further data analysis is required to translate the raw data into the corresponding electropherogram.

The 8 exons of the AR gene, labelled with the letters A, B, C, D, E, F, G, H have been amplified with 12 couples of oligonucleotide primers that also allow highlighting possible mutations in the splicing sites. 5 µl of DNA are added with 20 µl of forward primer mix and 20 µl of reverse primer mix, 2 µl of Taq polymerase, 3 µl of distilled H₂O. The exon amplification program from B to H includes:

Steps	Temperature	Timer	N. cycles	
1	94°C	10 minutes	1	35 cycles
2	94°C	35 seconds	1	
3	60°C	35 seconds	1	
4	72°C	30 seconds	1	
5	72°C	10 minutes		

First exon, as larger in size, was amplified with four overlapping pairs of primers and in this case the amplification program is more complex.

The amplified were directly sequenced using dideoxy chain terminator cycle sequencing protocol (BigDye™) and ABI 3730 DNA Analyzer (Applied Biosystems). The mutation site

was sequenced in 200 normal male samples as a control. Multiple sequence alignment, editing and consensus sequences have been built using AutoAssembler software (Applied Biosystems).

Results and Discussion

The result coming from the karyotype investigation highlighted in all cases a regular male karyotype (Figure 7). The F.I.S.H. showed in all cases the presence of the gene SRY (Figure 8) on the Y chromosome and the gene for the androgen receptor on the X chromosome (Figure 9). The molecular analysis has confirmed the observation of the F.I.S.H. highlighting in all cases the presence of the SRY gene (Figure 10), thus excluding the presence of the SRY gene on the X chromosome (an event that may result from an unequal crossing over between X and Y chromosomes) and leading to the conclusion of "male pseudohermaphroditism".

It should be noted that the molecular survey showed that the AZF region is intact (Figure 10).

The gene sequencing for the androgen receptor (AR) has shown the following:

Case 1: shows a nucleotidic substitution at the level of exon 3 in the position 2130 (AAA→TAA). This mutation generates the substitution of the amino acid lysine with a stop sequence in the 590 position in the amino acid chain. As a result there is lack of production of the androgen receptor, genetic condition associated with "complete androgen insensitivity syndrome" (C.A.I.S.)²³.

Case 2: shows a nucleotidic substitution at the level of exon 5 in the position 2559 (CAG→TAG). This mutation generates the substitution of the glutamic acid with a stop sequence in the 733 position in the amino acid chain. As a result there is lack of production of the androgen receptor, genetic condition associated with "complete androgen insensitivity syndrome" (C.A.I.S.)²⁴.

Case 3: shows a nucleotidic substitution at the level of exon 1 in the position 540 (CAG→TAG). This mutation generates the substitution of the glutamic acid with a stop sequence. As a result there is lack of produc-

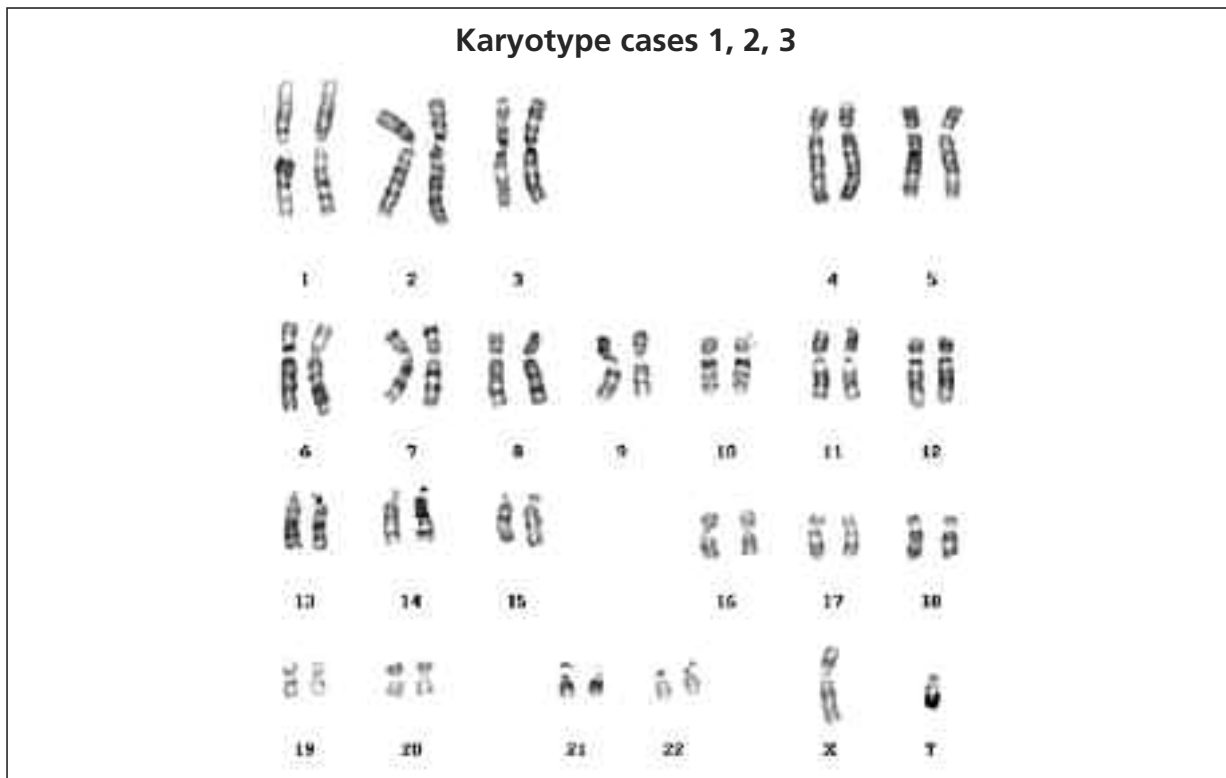


Figure 7. Normal male karyotype in patient with Morris syndrome.

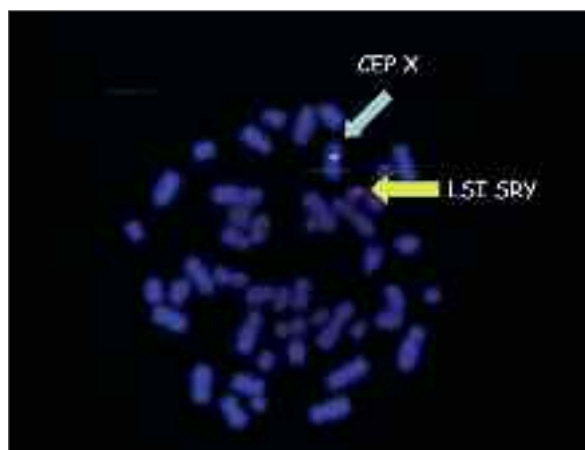


Figure 8. “LSI SRY” probe shows the presence of the SRY gene on the chromosome Y, while the “CEP X” probe highlights the centromere of chromosome X.

tion of the androgen receptor, genetic condition associated with “complete androgen insensitivity syndrome” (C.A.I.S.)²⁵.

All case studies presented a negative family history for CAIS.

Conclusions

Most cases (70%) of AR mutations are inherited and transmitted in an X-linked manner. In this situation, there is a likelihood of 50% for an XY offspring of being affected and for an XX off-

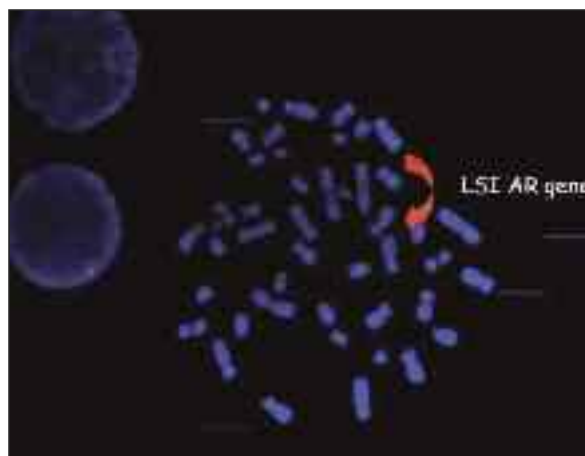


Figure 9. The presence of signal probe “LSI AR” on the chromosome X indicates the presence of the gene for the androgen receptor.

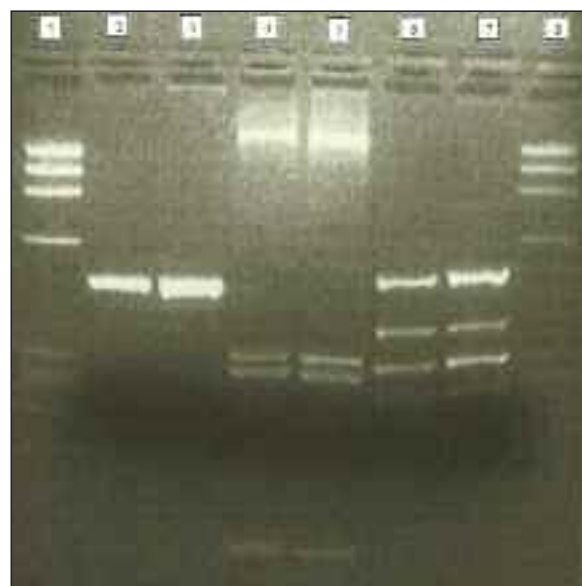


Figure 10. Lanes. 1, 8 = molecular weight; 2, 3 = Presence gene SRY (472 bp); 4, 5 = sY84 (326 bp), SY134 (301 bp), SY255 (126 bp); 6, 7 = ZFY (495 bp), sY254 (400 bp), sY86 (320 bp), sY127 (274 bp).

spring of being a healthy carrier. De novo mutations represent 30% of AR mutations and the risk of transmission can be considered as being very low, since there is to date no report of transmission to a second child. A rare exception to this (only two reported instances) is the case of germline de novo mutations in the mother. In this circumstance, the presence of germ cell mosaicism can be assumed and the risk of transmission is regarded as high²⁶⁻³¹. However, the possibility of germline mosaicism cannot be excluded in any case of de novo mutation of the AR gene and one should be cautious in the genetic counseling of these families.

The results of this work suggest the necessity of an adequate clinical and endocrinological study of patients with complete phenotype reversion from male to female in order to select precociously and more accurately the following laboratory tests that can allow defining the pathogenesis of this clinical condition in the majority of patients.

This study confirms the utility to perform an pelvic echographical examination in prepubertal and pubertal age to emphasize the regularity of the internal genitalia (uterus and ovaries). In the presence of a primary amenorrhea karyotyping is compulsory.

In case of suspicion of Morris syndrome^{32,33} is critical to find the rudimentary male gonads (using MRI, pelvic ultrasound, laparoscopy) and surgically remove them to prevent the onset of malignancies (teratoblastoma, gonadoblastoma)^{34,35}. Once established the disease in question, is useful for the patient and family have a continuous psychological help.

In the rare cases of newborn with ambiguous genitalia, a safe assignment of sex is impossible. It may lead to a real emergency situation, that is to be addressed quickly under the pressure of different and sometimes conflicting demands. The expert has to act quickly without making mistakes. Some findings are obvious and must be executed immediately: physical and instrumental (pelvic echography) examination, to examine chromosome, possibly preceded by research by F.I.S.H. signals specific to chromosomes X and Y in interphase nuclei, a molecular test for the presence or absence SRY gene, a dosage of 17-OH-Progesterone plasma. These findings may be useful to solve a certain number of doubtful cases. For more complex situations that reclaim more time to perform genetic testing and/or non-routine Enzyme assays shall be improved an interdisciplinary effort, to define the correct master sex of the newborn (not always coincident with the genetic sex, as for example in pseudohermaphroditism male with full sex reversal), without making mistakes that would later be very difficult to be correct.

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