CASE SERIES

THREE SIBLINGS WITH ANDROGEN INSENSITIVITY SYNDROME

Sisay Teklu, MD[1], Jakob Schneider, MD[2], Zewdu Terefework, MD[3], Benadam Shimeles, MD[1], Shemsu Abraham, MD[2], Endalk Bonsa, MD[1]

ABSTRACT

Genetic, gonadal, phenotypic and psychological genderis the basis for gender assignment to an individual. Derangement in genetic makeup, under or over exposure to sex hormones and problems related to sex hormone receptors will lead to abnormal development of the external and internal genitalia. Failure to respond for the endogenous androgen, Androgen Insensitivity Syndrome is one of the common causes of genital ambiguity and intersex. In this case series we have presented three girls from a family of seven children visited Tikur Anbassa Specialized Hospital (TASH) with a complaint of primary amenorrhea and diagnosed to have androgen insensitivity syndrome. Their clinical presentation, relevant laboratory and histopathologic findings, karyotype and genetic analysis results are summarized. Potential causes and treatment options are discussed.

Keyword: *Androgen Insensitivity Syndrome, Siblings*

INTRODUCTION

Androgen Insensitivity Syndrome (AIS) is typically characterized by evidence of feminization of the external genitalia at birth, secondary sexual development well below the age at puberty, and infertility in individuals with a 46 XY karyotype. The incidence of androgen insensitivity syndrome is estimated to be 1:20,000-64,000 male births(1).AIS is an X-linked recessive disorder (2). The androgen receptor (AR) gene is located on the X-chromosome at Xq11--12 and codes for a protein with a molecular mass of approximately 110 kDa. The androgen receptor belongs to the family of steroid-thyroid hormone-retinoid nuclear receptors. It contains 3 major domains: a hormone-binding region, a DNA-binding region, and an amino-terminal region. In the AR gene, four different types of mutations, ranging from single point mutation to complete gene deletion, have been detected in DNA from individuals with AIS, the most common being point mutation (3,4). This alteration in the gene blocks the body's response to androgen during fetal development and after birth.

AIS represents a spectrum of defects in androgen action and can be subdivided into three broad phenotypes: Complete androgen insensitivity syndrome (CAIS), with typical female external genitalia, partial androgen insensitivity syndrome (PAIS) with predominantly female, predominantly male, or ambiguous external genitalia and mild androgen insensitivity syndrome (MAIS) with typical male external genitalia (5). The gonad may be located in the abdomen, inguinal canal or external genitalia depending on the type of AIS. Complete androgen insensitivity syndrome was initially described by J.M. Morris, an American gynecologist in 1953 when he reported phenotypic female with 46 XY karyotype, presenting with primary amenorrhoea, adequate breast development and absent or scanty pubic and axillary hair and a vagina slightly shorter than normal that ends blindly (6). In Ethiopia AIS was first reported in the literature more than three decades ago (7).

Affected 46 XY individuals are almost always infertile. Each offspring of a female known to carry an *AR* pathogenic variant (heterozygous) have a 25% risk to become 46XY affected, 46XY unaffected, 46XX carrier and 46XX not carrier (8). There is relatively increased risk of malignant transformation of the

[1] Department of Obstetrics and Gynecology, School of Medicine, Addis Ababa University
[2] Department of Pathology, School of Medicine, Addis Ababa University
[3] CEO, Molecular Geneticist MRC-ET Molecular Diagnostics
* Corresponding author: siteet@yahoo.com , sisay.teklu1@aau.edu.et

gonads as age advances and removal of the gonads, where ever it is located, remains the main stay of prevention of malignant transformation after puberty. One report from Jima, Ethiopia, revealed seminoma of the intra-abdominal gonad in a case of AIS (9).

The estimated risk of gonadal malignancy in testicular feminization is 5%. In comparison to other intersex disorders, the premalignant risk is relatively low before puberty. However, the overall risk increases in patients older than 30 years and reaches up to 33% in patients above 50 years of age (10,11). The objective of this case report is to describe the genetic basis of AIS in these siblings and the importance of possible pre-conceptional genetic screening in families with this disorder.

PATIENTS AND METHODS

Medical charts of three sisters referred from eastern part of Ethiopia, Harare region to TASH, for complaint of primary amenorrhea was reviewed. Their demographic, clinical, laboratory, histo-pathologic, genetic analysis and treatment information was abstracted from the chart. The father of the three sisters was informed about the cases, potential genetic basis of the disease and consented for investigation, all possible treatment options and agreed to undergo genetic screening for the remaining siblings. He also permitted the authors to review patient charts and use investigation results for this case report. Histopathologic examination of the specimens was done at Addis Ababa University, School of Medicine, Department of Pathology and Genetic analysis was done at MRC-ET Molecular Diagnostics, Addis Ababa, Ethiopia.

Case 1: A 22 years old woman from Eastern Ethiopia, Harar, was brought to TASH by her father for complaints of primary amenorrhea and left inguinal swelling. She had right side inguinal surgery at the age of three for inguinal mass at a local hospital. She is not married and never had sexual contact. She is the fifth child for the family. Her childhood was not different from her siblings and she has completed high school.

Case 2: A 20 years old, who is the sixth child for the family, was brought to TASH by her father for a complaint of primary amenorrhea and bilateral inguinal swellings from eastern part of Ethiopia, Harar. Her childhood was not remarkably different from her siblings and has completed high school.

Case 3: A 15 years old girl, who is the seventh child for the family, was brought by her father because the father recognized delayed menarche and bilateral inguinal swelling since childhood. He was afraid that she may also not have menses in the future like her two older sisters (cases 1 and 2).

Their mother does not have any chronic medical illness, habits or allergy. She did not have any problem during the past seven pregnancies; all were home deliveries and were normal. She never had any disease or exposure to drugs during pregnancy.

According to the father, the first three children are female, had menarche on average at the age of 14, and had normal linear growth for age, pubic and axillary hair growth at the appropriate age. All three got married at about the age of 17 and all three gave birth and are currently in good health. The fourth child is a male, normal childhood growth and development, married and fathered two children. The father is now age 65 years, sexually active and in good health.

The three cases in this case report underwent a complete physical examination and laboratory investigations (see Table 1). After confirming that pubertal development is achieved, father was told about the problem and counseled about the available treatment options including hormone replacement and the girls' future family and reproductive life. Surgical removal of the inguinal masses and hernioraphy was done under local anesthesia (2% Lidocain infiltration) in the major operating theater. All the three cases had smooth post-operative course and estrogen replacement was given during the post-operative follow up period to maintain the female phenotype.

Gross and Microscopic pictures of the specimens: The gross features of the removed masses are strikingly similar: small testicles with gray white glistening surface and a solid whitish mass at one pole. Also the cut surface is similar in all specimens and reminiscent of a small testicle with whitish tunica albuginea and pale yellowish parenchyma. The only difference being in the parenchyma of the second case there were small brighter foci with a diameter of up to 1cm (Figure 1).

The common microscopic features of the gonads of all three patients are: typical immature tubuli seminiferi, variable sized, most completely filled with Sertoli cells, few with a small central lumen, and scattered spermatogonia. None with features of intratubular germ cell neoplasia. In all, the interstitial leydig cells are abundant (figure 2). Within this back-

ground each testicle contains well delimited nodes consisting of similar tubuli which are crowded and not surrounded by interstitial tissue with Leydig cells (figure 3 a, b, c). The node at one pole of the testicle consists of bundles of smooth muscle cells accompanied by collagen fibers. Based on these findings a diagnosis of immature cryptorchicd testicles with Sertoli cell hamartoma and degenerative changes is made. Differences in the specimens of the three cases are the number and size of Sertoli cell hamartomas, degenerative changes and absence of spermatogonia in case 1, very few spermatogonia with no maturation in case 2 and few spermatogonia with no maturation in case 1.

Table 1: Summary of clinical and laboratory findings of the cases

Clinical and histopathology	Case 1	Case 2	Case 3
Age	22	20	15
Birth order	5th child	6th child	7th child
Assigned gender	Female	Female	Female
Complaints	Primary amenorrhea and inguinal swelling	Primary amenorrhea and inguinal swelling	Primary amenorrhea and inguinal swelling
Height(Cm)	178	174	169
Weight(Kg)	73	64	57
Breast Tanner stage	Stage V	Stage V	Stage IV
Abdominal examination	Rt inguinal old surgical scar, 2×2 cm mobile, firm Lt inguinal mass	Bilateral inguinal mass mobile, firm and non tender, 2×3 cm	Bilateral inguinal mass mobile, firm and non tender, 2×3 cm
Axillary hair	Absent	Absent	Absent
Pubic hair	Stage I	Stage I	Stage I
Clitoris	Absent	Present	Present
Labia majora/ minora	Present	Present	Present
Urethral opening	Normal	Normal	Normal
Vaginal canal	Short but well developed	Short but well developed	Short but well developed
Cervix by Ultrasound	Absent	Absent	Absent
Uterus by Ultrasound	Absent	Absent	Absent
Ovary by Ultrasound	Absent	Absent	Absent
Serum FSH(mu/ml)	8.3	1.5	11.4
Serum LH (NV 1.1-7.0 mu/ml)	10.4	11	41
Serum estrogen(pg/ml)	87.3	50.61	50.87
Serum testestron (NV 0.1-0.9ng/ml)	6.9	11.62	8.72
Karyo type	46XY	46XY	46XY
Treatment given	Lt side gonadectomy and hernioraphy at the age of 22 years	Bilatteral gonadectomy and hernioraphy at the age of 20 years	Bilatteral Gonadectomy and hernioraphy at the age of 15 years

Figure 1. Bilateral gonads of case 2 with multiple well delimited brighter foci on the cut surface. Whitish node at the upper pole consisting of connective tissue (Collagen and smooth muscle).
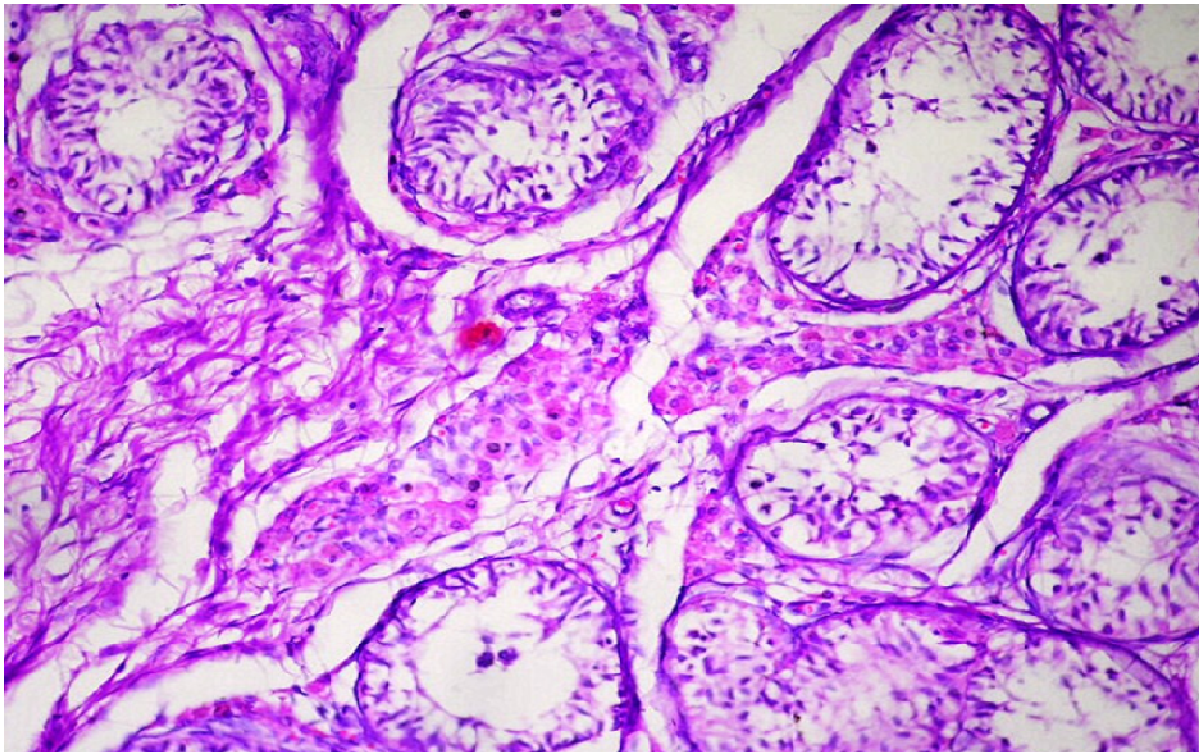

Figure 2. Testicular parenchyma similar in all specimens: Tubuliseminiferi filled with Sertoli cells and few spermatogonia (larger cells with clear cytoplasm). Interstitial tissue with Leydig cell hyperplasia.
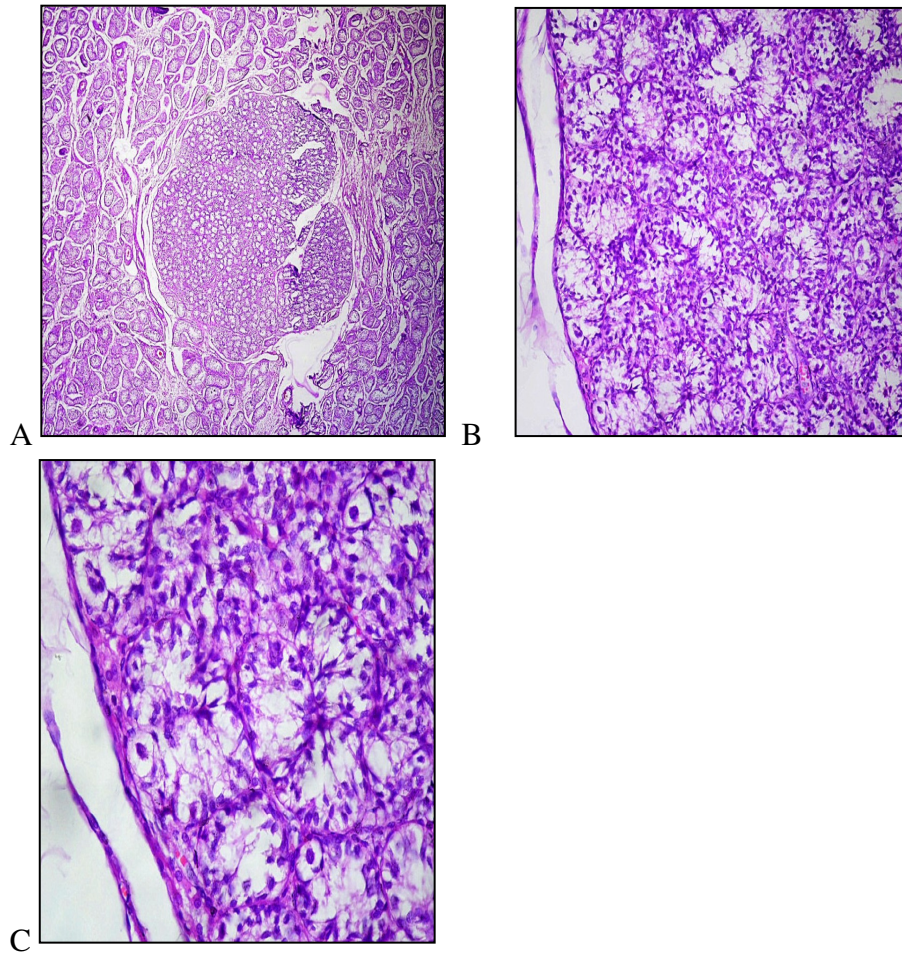
Figure 3. A, B well delimited nodule of crowded seminiferous tubuli without intervening interstitium
C. High power view of the tubuli filled with Sertoli cells and few spermatogonia (large nucleus
and abundant clear cytoplasm). No interstitial Leyig cells.
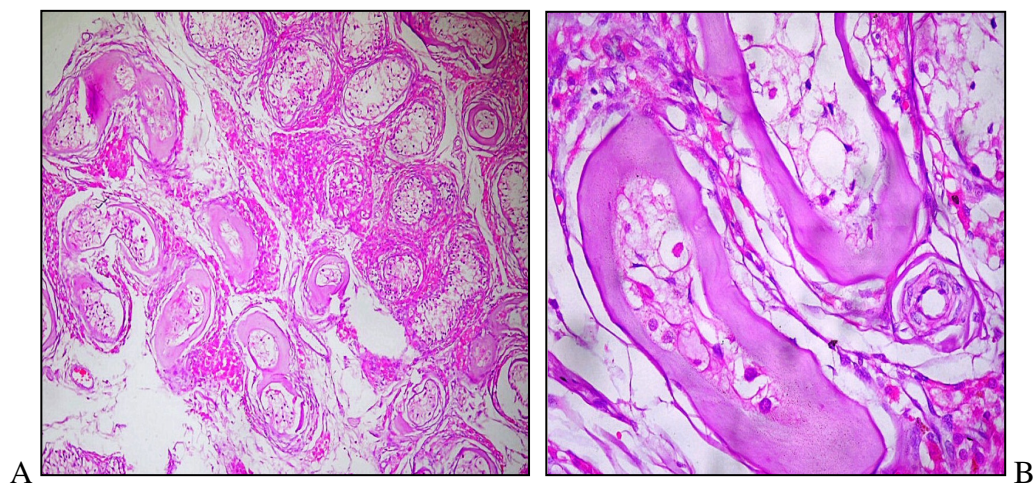

Figure 4. A. Expanded interstitium and scattered tubuli with thickened and hyalinized
laminapropria B. High power view of tubuli with marked hyalinization.

Genetic Analysis of the three cases: The presence of genetic anomalies and the karyotype was investigated by the MLPA method (Schouten et al. 2002), following the one tube protocol (www.mlpa.com). MLPA is a multiplex PCR method that allows amplification of multiple targets on a genome in a single PCR reaction with only a single primer pair. The relative ease of use, affordability, and generation of fast and easy to interpret data makes MLPA very useful to study copy number variations, insertions, deletions and duplications of genomic regions. "MLPA identifies exonic or whole gene deletions and duplications not detectable by sequence analysis" (Gottlieb et al. 2014). However, sequence analysis is a preferred method of choice for investigating genetic alterations of clinical significance that are caused by point mutations and/or single nucleotide polymorphisms.

DNA was extracted from whole blood and paraffin embedded tissue using Invitek DNA extraction kit (Thistle Scientific, UK) according to the manufacturers' instruction manual. The DNA isolation from paraffin embedded tissue was done using a slight modification of the Invitek protocol in which 0.5cm tissue sample was heated at 90ºC for 15min to melt the paraffin in the presence of 200µl lysis buffer. The lysis process was then continued overnight at 56ºC after adding 20µl of proteinase K (15µg/ml). The Proteinase K was inactivated by heating at 98ºC for 15 min. Purification of the lysate and DNA elution was then accomplished by passing the lysate through the RTA spin filter with subsequent binding and washing using the buffers included in the kit. 50ng of DNA was then used as a template for the MLPA procedure.

The kits, provided by MRC-Holland, the Netherlands, used for MLPA reaction were: P050 (Congenital Adrenal Hyperplasia), P074 (Androgen Insensitivity Syndrome (AIS), P0185 (Intersex) and P0360 (Y chromosome). Briefly, the MLPA protocol includes a 16hr hybridization of probes targeting specific regions in the genome. The adjacent probes were then ligated and then amplified by multiplex PCR with one set of universal primers that were part of the probe. The data was analyzed using Coffalyer.Net software (MRC-Holland, The Netherlands).

The results show a 46XY karyotype for all samples. Screening of the subtelomeric regions of all chromosomes did not reveal gross chromosomal abnormality and ploidy error was not observed in any of the samples. The sex chromosomes X and Y did not show anomalies and the SRY region did not appear to be deleted or duplicated. The Y chromosome analysis kit is designed to detect Y chromosome microdeletions on three specific regions that are located on the long arm of the Y chromosome. All the DNA probes from these regions showed clearly discernible signals confirming the presence of Y chromosome in all cases. The analysis on the 9 exons of the AR gene did not show copy number variation. The MLPA kit used in this study is designed to detect deletions/ duplications of one or more sequences in the AR gene in a DNA sample. It is to be noted that most defects in this gene are expected to be small (point) mutations, most of which will not be detected by MLPA. Therefore, unaccounted mutations on the AR genes could be present. Sequence analysis could reveal more information and explain the clinical scenario better.

DISCUSSION

Male external and internal genital development requires optimal androgen exposure during intra uterine development and after delivery. Presence of adequate level of androgen hormone and AR are vital for normal genital differentiation. AR genes codes for androgen receptors. Most cases of AIS are due to failure of normal male sexual differentiation in a person with 46 XY karyotype because of defective androgen receptors due to AR gene mutation. Until 2010, more than 400 mutations of this gene have been discovered and they are either inherited from the mother, in an X-linked recessive pattern, or are *de novo* spontaneous mutations (12). Therefore individuals with 46 XY karyotype, carrying the defective gene manifest the disorder. The diagnosis of AIS in individuals with a 46 XY karyotype is based on the following clinical findings: undermasculinization of the external genitalia, impaired spermatogenesis with otherwise normal testes, absent or rudimentary müllerian structures, evidence of normal or increased synthesis of testosterone and its normal conversion to dihydrotestosterone, and normal or increased luteinizing hormone (LH) by the pituitary gland (8).

Our cases are the last three consecutive siblings from a family of seven. The parents have noticed inguinal region swelling during child hood in the first case and she was taken to health facility where one side inguinal mass was removed. The absence of menses at puberty and the presence of inguinal mass in the last three daughters was the main concern in this family as is the case in many of the cases in the lit-

erature (9). The first three of the seven siblings in this family are female, have normal complete female pubertal development, married and gave birth while the fourth sibling is normal fertile male. This suggests that in this family the defective gene probably was carried by the mother in one of the X chromosomes and each offspring were having a 25% risk to become 46XY affected, 46XY unaffected, 46XX carrier and 46XX not carrier, as described in the literatures (8).

The three fertile sisters in this family need to have genetic screening for AR mutation to identify those carrying the defective gene. In the three cases described in this report, histologic evidence of inguinal testis and 46XY karyotype in phenotypic female confirm the diagnosis of AIS. The presence of blind ending vagina, well developed breasts and absent pubic and axillary hair growth together with absent mullerian derivatives by ultrasound in our cases suggest CAIS. Although the diagnosis of AIS is confirmed with the identification of a molecular defect in the AR gene, genetic analysis from tissue and blood sample in our cases did not show any copy number variation in the 9 exones of AR gene. This may be because the technique we used (MLPA) did not detect small point AR gene mutations or may be because there are cases of Testicular Feminization that occur without a mutated androgen receptor gene. Other much less frequent causes are: mutant steroidogenic factor-1 protein; a deficit in the transmission of a transactivating signal from the N-terminal region of the normal androgen receptor to the basal transcription machinery of the cell (13).

Endocrine features of AIS (complete or partial) may show normal or high levels of serum Luteinizing Hormone (LH) and Testosterone (T) during the first three months of life, normal range until puberty, elevated serum levels after puberty due to the androgen insensitivity and the consequent lack of negative feedback exerted by sex hormone on hypothalamus and hypophysis (14). The physical findings and hormonal profile of our cases go for post pubertal CAIS.

Gonads may be located in the abdomen, inguinal canal or may descend further in to labia majora depending on the extent of the androgen insensitivity. Invariably in all cases of AIS, histopathologic examination of the gonads shows features of Sertoli-Leydig cell suggestive of testis with or without neoplastic changes (10).

The specimen from our cases showed histologic features of testis with some hamartomatous changes, which is benign with no evidence of malignancy. Few germ cells were seen with no maturation in the youngest while there was no any germ cell seen in case 1 suggesting that cases of CAIS are invariably infertile. These gonads are the primary source of sex hormones, estrogen (after aromatization of excess androgen), required for development of secondary sexual characteristics. The chance of malignant transformation of the gonad before puberty is very low and it is not recommended to remove the gonads before puberty. After puberty, since the risk of malignancy markedly increases, it is recommended to remove the gonads and supplement estrogen to maintain and support female phenotypic secondary sexual characteristics (10, 11).

Our cases undergone gonadectomy after confirming complete pubertal development to prevent potential malignant transformation in the future. Counseling is the most important part of management of patients with AIS. Pre-conceptional counseling and testing in families with history of AIS, if supplemented with genetic screening of their fetuses during early pregnancy, will help identify 46XX mothers carrying mutant AR gene and phenotypic female fetuses with 46XY karyotype, hence can prevent delivery of a baby with AIS (15).

ACKNOWLEDGEMENTS

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