The Egyptian Journal of Medical Human Genetics (2015) **16**, 245–256

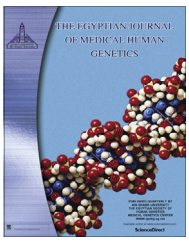
HOSTED BY

ELSEVIER

Ain Shams University

The Egyptian Journal of Medical Human Genetics

www.ejmhg.eg.net
www.sciencedirect.com



ORIGINAL ARTICLE

Molecular study of developmental sex disorders in children

Soheir S. AboElella, Maha A.M. Tawfik *, Wafaa moustafa M. Abo El-fotoh

Department of Pediatrics, Faculty of Medicine, Menoufiya University, Egypt

Received 15 January 2015; accepted 10 February 2015
Available online 7 March 2015

KEYWORDS

DSD;
Ambiguous genitalia;
SRY;
SOX9;
NR5A1;
Genetic counseling

Abstract *Background:* Sex determination and differentiation in humans are processes that involve the interaction of several genes such as SRY, SOX9 genes which are important in the development of the male genital system. Also NR5A1 gene plays an important role in the development of gonads and the adrenal glands. Aim of the study include clinical assessment of children with disorders of sex development, molecular analyses for SRY, SOX9 and NR5A1 genes and genetic counseling for the patients and their families.

Subjects and methods: This study included sixteen patients from 1 day to 6 years old attending the Genetics and Endocrinology unit, Pediatric department, Faculty of Medicine, Menoufiya University, Egypt. All cases were subjected to: detailed history, thorough clinical examination, routine and hormonal investigations, imaging studies, cytogenetic and molecular studies for SRY, SOX9 and NR5A1 genes.

Results: Positive consanguinity between the parents was detected in seven patients (43.75%). Serum 17 OH progesterone was elevated in five patients (31.25%) and below normal ranges in two patients (12.5%). Cytogenetic study revealed six patients with normal (46, XX) karyotype, eight patients with normal (46, XY) karyotype, one patient had (45, X) karyotype and another with (45, X/46, XY) karyotype. Thirteen out of sixteen patients undergone molecular studies, SRY gene was +ve for six patients with normal male (46, XY) undergone and one patient with (45, X) karyotype (translocated SRY). SRY was −ve for five patients with normal female (46, XX) karyotype and one patient with (45, X/46, XY) karyotype (deleted SRY). All patients were +ve for SOX9 and NR5A1 genes and no deletions detected.

Conclusion: Genetic studies beside clinical and hormonal evaluation will allow us to rapidly reach a diagnosis and to identify a 'molecular sex' for each patient.

1. Introduction

Molecular mechanisms of sex determination involve a growing network of genes, a large number of which are transcription factors. The transcription factors so far identified as having a major role in sex determination, namely SRY, SOX9 and

* Corresponding author. Tel.: +20 034252090.
E-mail address: amkthabet@yahoo.com (M.A.M. Tawfik).
Peer review under responsibility of Ain Shams University.

http://dx.doi.org/10.1016/j.ejmhg.2015.02.005

NR5A1, can be disrupted by a number of mutations that can affect either their availability in the nucleus or their function [1].

Steroidogenic factor 1 (SF1), denominated as nuclear receptor subfamily 5 group A member 1 is a protein that regulates several steps of adrenal and gonadal development. It is encoded by the NR5A1 gene, which is an autosomal gene mapped to 30 kb within 9q33. NR5A1 is expressed in the developing urogenital ridge, steroidogenic tissues (such as gonads, adrenals, and placenta), hypothalamus and anterior pituitary [2].

In general, it activates the expression of anti-mullerian hormone (AMH) in sertoli cells leading to the regression of Müllerian structures and in leydig cells, it activates the expression of several enzymes involved in steroidogenesis, resulting in the virilization of external genitalia and testicular descent. In ovaries, NR5A1 is expressed in the granulosa and theca cells where it regulates genes required for ovarian steroidogenesis and follicle growth maturation [3]. Therefore, mutations in NR5A1 may lead to disorders of sex development (DSD) defined as incomplete or disordered gonadal or genital development, causing divergences between genetic sex, gonadal sex and phenotypic sex [4].

The objective of this study is clinical assessment of children with ambiguous genitalia, molecular analyses for SRY, SOX9 and NR5A1 genes and genetic counseling for the patients and their families.

2. Patients and Methods

2.1. Patients

The study was conducted on sixteen patients with ambiguous genitalia. They were selected from Genetics and Endocrine Unit, Pediatric department, Menoufiya university hospitals, Egypt. They were seven apparent female patients and nine apparent male patients. Their ages ranged from the 1 day to 6 years old.

2.2. Methods

All studied patients were subjected to the following:

1. A detailed history including maternal diseases resulting in virilization as (maternal luteomas), exposure to androgens and endocrine disrupters (phenytoin). Consanguinity, recurrent miscarriages, unexplained infant death and neonatal history of failure to thrive, vomiting, skin pigmentations and progressive virilisation were reported [5].
2. A thorough physical examination of studied patients (vital signs, anthropometric measurements, other congenital abnormalities, dysmorphic features, hirsutism or abnormal body pigmentations) was also reported [6].
3. A careful examination of the external genitalia of studied infants includes:-
   a. Size of phallus.
   b. Examination for the testes in apparent male patients (palpable or not, their size, location, and texture).
   c. Prader score application for degree of virilization in apparent female cases [7].
   d. Calculating the External Masculinisation Score (EMS) in male cases [8].
4. Tanner staging system for pubertal changes [9].
5. Routine investigations included complete blood picture, serum electrolytes especially (Na and K) and glucose levels for cases with salt losing congenital adrenal hyperplasia (CAH) [10].
6. Hormonal studies involved serum levels of 17 hydroxy progesterone, cortisol, adrenocorticotropic hormone, testosterone, dihydrotestosterone, dehydroepiandrosterone, and androstendione according to suspected diagnosis and interpreted in relation to specific reference ranges [11].
7. Imaging studies including abdominopelvic ultrasonography, CT and or MRI for visualization of the internal genitalia, gonadal site and structure if present. [12].
8. Genetic studies including
   a. Cytogenetic analysis (chromosomal karyotyping) was performed on peripheral blood from the patients' lymphocytes that were cultured according to the standard method. Banding patterns were analyzed [13]
   b. Molecular studies for detection of absence or presence of gene products for SRY, SOX9 and NR5A1 genes. The following steps were applied:
      1. DNA extraction from blood samples: Genomic DNA was extracted from peripheral blood samples using Thermo Scientific GeneJET™ PCR Purification Kit (# K0721 made in EU. Lithuania) that utilizes a proprietary silica-based membrane technology in the form of a convenient spin column according to manufacturer's instructions.
      2. Polymerase Chain Reaction: Each PCR reaction was performed with mixture consisted of 0.2 µg of genomic DNA, 2.5 U of Taq DNA polymerase, 0.5 µmol/L of each primer, 100 mmol/L dNTPs, 3.0 mM $MgCl^2$ and 1× PCR buffer in a final volume of 50 µl. The PCR conditions were 5 min at 94 °C for pre-heating, 35 cycles of 94 °C for 30 seconds, 57 °C for 30 seconds and 72 °C for 1 min, and 72 °C for 10 min post-extension using an automated thermal cycler (Bio-Rad MJ Research PTC-200 DNA Engine thermal cycler).
      3. Primer sequences of studied genes were as follows:

Gene	Forward primer	Reverse primer
SRY	5′-GAATATTCCCG CTCTCCGGAG-3′	5′-ACCTGTTGT CCAGTTGCACT-3′
SOX9	5′-TATGACTGG ACCCTGGTG-3′	5′-TGTGGCTTGT TCTTGCTGG-3′
NR5A1	5′-CCGCAGCATT ACCAACACCACA-3′	5′-CATCCGTGCT TTATCCTGAGCTG-3′

4. Reaction products were electrophoresed on 3% agarose–TBE gels containing 0.5 µg/ml ethidium bromide and DNA bands visualized under UV light, and their positions determined in relation to DNA ladders (Thermo Scientific GeneRuler 50 bp and 100 bp DNA Ladders).
9. Genetic counseling was done for all families including clinical aspects and diagnostic approach about the child condition with the parents, information on available options and determining methods for communication and follow up.

10. Informed consent was taken from parents of the children. The study was approved by the ethical committee of Menoufiya University. The work has been carried out in accordance with the code of Ethics of the World Medical Association (Declaration of Helsinki) for experiments in humans.

3. Results

The present study includes sixteen patients aged from 1 day to 6 years. Their presenting manifestations varied between ambiguous genitalia, salt losing attacks, abnormal body pigmentations and hair growth and urine stream abnormalities.

Demographic data of studied patients: seven patients (43.75%) had consanguineous parents. In addition, patients (14 & 15) were cousins. Five patients (31.25%) had possible similar conditions in their families (Table 1). Anthropometric measurements showed that four patients (25%) were below 3rd percentile for weight and below < -2 SD for BMI and three of them were below 3rd percentile for length.

The external genital manifestations of the female patients (Table 2) were mainly clitoromegaly and fused labia, with no palpable gonads and two of them had abnormal pubic hair growth. Prader score for apparent female patients: three had score of 1, two had score of 2 and another two patients had score of 4. The external genital manifestations of the male patients were mainly bifid scrotum and urethral opening at phallus base. Three had microphallus. Two had only one palpable gonad and one patient number (12) looked apparently female (Table 3).

External masculinization score was applied to male patients, and all nine patients were involved and their total scores were below normal score ($\geqslant 11$). Tanner staging system showed that all patients are in 1st pubertal stage except two female patients (4 & 16) who were with pubic hair stage 2.

Serum electrolytes and blood glucose levels were within normal levels in all patients except in three patients (2, 12 & 14) who had hyperkalemia and hyponatremia, and only two of them (12 & 14) suffered from hypoglycemia.

Hormonal evaluation revealed that five patients (31.25%) had above normal ranges for serum 17 OH progesterone levels and two (12.5%) had below normal ranges. From these seven patients, six had elevated serum ACTH levels and low serum cortisol levels. Mean serum 17 OH progesterone level for all cases was: 321.25 ± 603.57 ng/dl SD. Mean serum ACTH levels were, Am: 101.56 ± 88.2 pg/ml, Pm: 59.3 ± 51.98 pg/ml. Mean serum cortisol levels were, Am: 11.1 ± 8.31 ug/dl, Pm: 10.87 ± 7.5 ug/dl.

Imaging studies revealed that all genetic female patients had internal female genitalia except patient (6) with (45, X) karyotype. Clinically, this patient had bifid scrotum with palpable right testis and his phallus was 2.5 cm in length and imaging studies showed no internal female genitalia, right scrotal testis and left inguinal one.

All genetic male patients showed no internal female genitalia except patient 7 with 45, X/46, XY karyotype who showed uterus and bilateral pelvic streak gonads. Clinically, this patient was an apparent female on external genitalia with clitoromegaly, fused labia, identified vaginal and urethral orifices and no palpable gonads (tables 2 and 3).

Cytogenetic studies showed six female patients with (46, XX) DSD, eight male patients with (46, XY) DSD, one had 45, X and another with 45, X/46, XY.

Regarding molecular studies, SRY products were detected at 418 bp position and SOX9 products at 270 bp compared to 100 bp DNA ladder on gel electrophoresis. NR5A1 products were detected at 50 bp compared to 50 bp DNA ladder on gel electrophoresis. We must note that the molecular studies were performed for all patients except patients 4, 11 and 13 who refused sample withdrawal.

Results for SRY gene revealed that patient 6 with 45, X karyotype had a translocated SRY gene and patient 7 with 45, X/46, XY karyotype had a deleted SRY (Figs. 3 and 4). SOX9 and NR5A1 genes were present in all studied patients and no deletions detected (Figs. 5–8).

Genetic counseling was done through frequent sessions and included:

1. Discussing clinical aspects and diagnostic approach about the child's condition with the parents and its importance before rushing to gender selection.
2. Documentation of family history and pedigree information.
3. Consent for photography and blood sample withdrawal.
4. Recognition of inheritance patterns and risk estimation about the disease if with genetic basis.
5. Information of available options to help making informed medical and personal decisions.
6. Determining methods for communication and follow up.

4. Discussion

In the present study the ages of our patients ranged from 1 day to 6 years old with mean for age of presentation 2.2 ± 3.3 months, the commonest age of presentation was the neonatal period (11 cases; 68.75%), followed by infancy with five cases (31.25%). In agreement to our results, Hughes [14] reported that DSD typically are diagnosed at birth in infants with ambiguous genitalia while, disorders associated with phenotypic males and females may be diagnosed much later.

Five patients had possible similar conditions in their families and two of them were cousins. This was consistent with the approach taken by Clayton et al. [15], who documenting that a family pedigree is an essential step in management of DSD and associated genetic counseling.

Seven patients (43.75%) had a positive consanguineous parent and patients (14 & 15) were cousins. This was consistent with a study conducted by Mazen et al. [16] which showed consanguinity rate of 61% in the affected families with DSD. Not forgetting that CAH is autosomal recessive, so the high prevalence of consanguinity in our cases is a significant risk factor in DSD cases, which was also reported by Nimkarn [17].

Presenting manifestations included ambiguous genitalia, salt-losing crisis in three patients, abnormal body hair growth in two female patients, abnormal body pigmentations in one female patient and abnormal urine stream in two apparent male patients. These presentations were in line with Warne and Raza [18] who reported that the signs of DSD vary from being not recognized at all as in cases with non salt-losing CAH, to being completely ambiguous external genitalia.

Table 1 Demographic data of the studied patients.

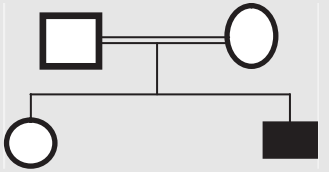
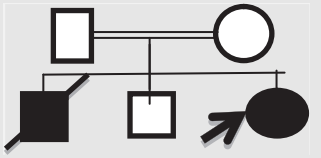
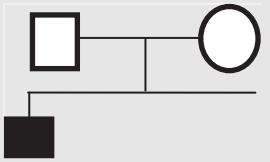
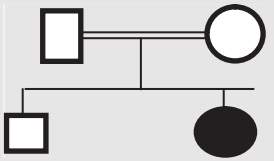
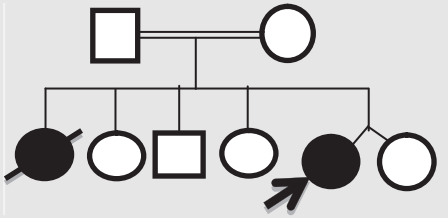
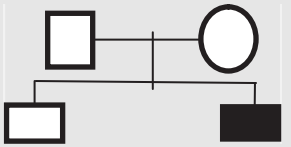
Serial No.	Age at examination	Age of clinical presentation	Birth order	Maternal age (yrs)	Paternal age (yrs)	Consanguinity	Maternal and obstetric history	Family history	Pedigree
1	2 yrs	1 day	2nd	23	27	+ve			
2	4 mth	1 day	3rd	25	26	+ve		1st sibling died at 6 months old with vomiting attack (similar condition)	
3	2 mth	1 day	1st	20	32	−ve			
4	30 mth	5 mth	2nd	23	33.5	+ve			
5	10 mth	3 mth	5th	38	49	+ve		1st sibling died at age of 3 years with similar condition	
6	1 day	1 day	2nd	27	32	−ve			

Table 1 Demographic data of the studied patients.

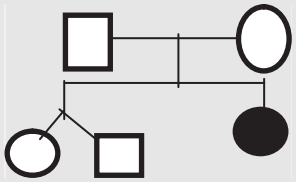
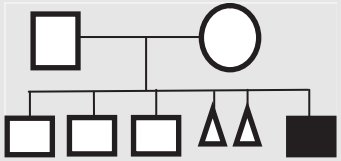
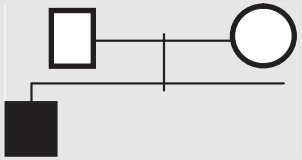
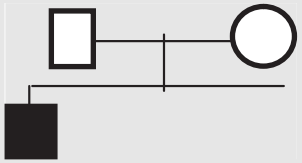
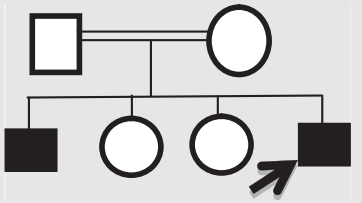
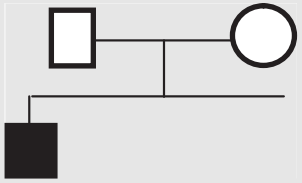
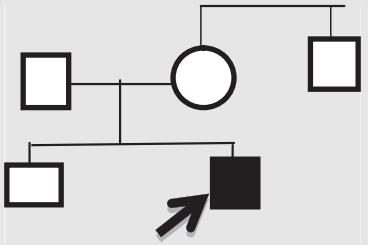
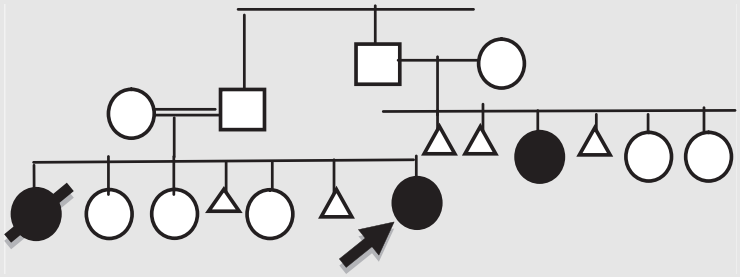
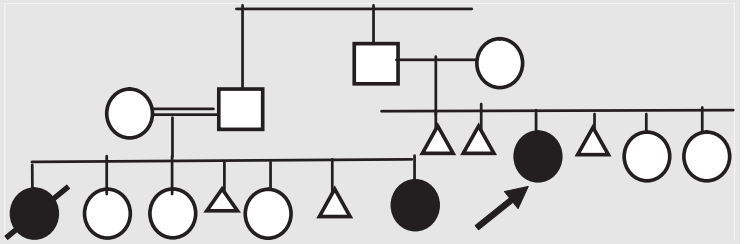
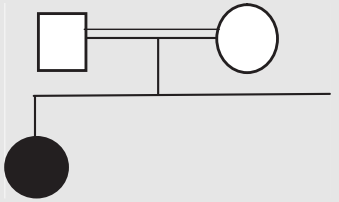
Serial No.	Age at examination	Age of clinical presentation	Birth order	Maternal age (yrs)	Paternal age (yrs)	Consanguinity	Maternal and obstetric history	Family history	Pedigree
7	18 mth	1 day	3rd	29.5	32.5	−ve			
8	20 mth	1 day	4th	40	46	−ve	Previous 2 abortions.		
9	30 days	1 day	1st	25	31	−ve			
10	1 day	1 day	1st	22	25	−ve			
11	1 day	1 day	4th	36	40	+ve		Similar condition in his elder brother	
12	1 yr	3 wk	1st	20	25	−ve			

Table 1 Demographic data of the studied patients.

Serial No.	Age at examination	Age of clinical presentation	Birth order	Maternal age (yrs)	Paternal age (yrs)	Consanguinity	Maternal and obstetric history	Family history	Pedigree
13	2 days	1 day	2nd	23	28	−ve			
14	5 mth	4.5 mth	5th	37	41	+ ve	Previous 2 abortions.	– Dead elder sister by a similar condition and living cousin. –Three abortions in her cousin's mother	
15	6 yrs	4 mth	1st	30	36	−ve	Previous 3 abortions, two before and one after her birth	– One living cousin and another dead one. – Two abortions with her cousin's mother.	
16	1 yr	11 mth	1st	19	28	+ ve			

mth, month; yrs, years.

Table 2 Data summary of apparent female patients: (7 patients).

Serial No.	Presentation diagnosis	Local examination	Serum K level	Hormonal investigations						Imaging studies	Karyotyping	SRY gene	SOX9and NR5A1 genes	Diagnosis
				ACTH (pg/ml)		Cortisol (μg/dl)		17 OHP (ng/dl)	Others					
				Am	Pm	Am	Pm							
2	Ambiguous genitalia and recurrent attacks of vomiting	Genitalia shows – Clitoromegaly (3.75 cm) – Fused labia with single vestibule – No palpable gonads	5.5 ↑	215 ↑	78 ↑	1.6 ↓	1.9 ↓	2000 ↑	DHEA 84.5 ng/dL ↑	Internal female genital organs	46 XX	−ve	+ve	CAH
4	Large clitoris and abnormal hair growth	Genitalia shows – Clitoromegaly (3.5 cm) – Fused labia with identified urethral and vaginal orifices, – Scanty pubic hair – No palpable gonads	5	166 ↑	92 ↑	1.5 ↓	2.1 ↓	358 ↑	DHEA 87 ng/dL ↑ and androstendione 53 ng/dl ↑	Internal female genital organs and ovaries	46 XX	–	–	CAH
5	Large clitoris	Genitalia shows – Clitoromegaly(2.7 cm) – Fused labia with urethral opening like penile hypospadias – No palpable gonads	3.7	264 ↑	115 ↑	1.4 ↓	1.7 ↓	1684 ↑	Androstendione 47.3 ng/dl ↑ and free testosterone 0.4 ng/dl ↑	Internal female genital organs	46 XX	−ve	+ve	CAH
7	Large clitoris	Genitalia shows – Clitoromegaly(3 cm) – Fused labia with identified vaginal and urethral orifices – No palpable gonads	4.5	29	18	10.5	11.6	35		Uterus and ectopic kidneyand bilateral steak gonads	45X/46 XY	−ve	+ve	Mixed gonadal dysgenesis
14	Dehydration attack	Genitalia shows – Clitoromegaly (1.3 cm) – No fused labia with identified vaginal and urethral orifices – No palpable gonads	6 ↑	267 ↑	217 ↑	1.4 ↓	1.8 ↓	10 ↓	DHEA 96 ng/dL ↑ and androstendione 3.5 ng/dl ↓	Internal female genital organs	46 XX	−ve	+ve	CAH
15	Abnormal body pigmentations and clitoromegaly	Genitalia shows – Clitoromegaly (1.6 cm) – No fused labia with identified vaginal and urethral orifices – No palpable gonads	3.9	136 ↑	75 ↑	1.9 ↓	2.2 ↓	254 ↑	DHEA 152 ng/dL ↑	Internal female genital organs	46 XX	−ve	+ve	CAH
16	Abnormal body hair growth	Genitalia shows – Clitoris about 0.9 cm in length – No fused labia, with identified vaginal and urethral orifices – and no palpable gonads	4.7	28	31	19.4	17.6	32	Normal DHEA and androstendione levels	Internal female genital organs	46 XX	−ve	+ve	Idiopathic hirsutism

CAH; congenital adrenal hyperplasia, 17 OHP; 17 OH progesterone.

Table 3 Data summary of apparent male patients: (9 patients).

Serial No.	Presentation	Local examination	Hormonal assays						Imaging studies	Karyotyping	SRY gene	SOX9 and NR5A1F gene	Diagnosis
			ACTH (pg/ml)		Cortisol (μg/dl)		17 OHP (ng/dl)	Others					
			Am	Pm	Am	Pm							
1	Ambiguous genitalia	Genitalia shows – 2 palpable gonads – Phallus about 3 cm in length – Bifid scrotum with urethral opening at phallus base	44	27	11.7	13.1	198 ↑	DHEA 14 ng/dl ↓, androstendione 3.5 ng/dl ↓ and free testosterone 0.16 ng/dl ↓	No internal female organs and bilateral labioscrotal gonads	46 XY	+ ve	+ ve	CAH
3	Ambiguous genitalia	Genitalia shows – 2 palpable gonads (1x 1 cm) – Phallus about 2.75 cm from ventral aspect – Bifid scrotum with hypospadias	33	21	15	17	66	Free testosterone 2.16 ng/dl ↑ and Total 326 ng/dl ↑, DHT 0.3 ng/dl ↓ T: DHT ratio 7.2 and after hCG was 37.4	No internal female genital organs and bilateral labioscrotal gonads	46 XY	+ ve	+ ve	5 α reductase deficiency
6	Ambiguous genitalia	Genitalia shows – Palpable Rt testis (1.5 x 1 cm) – Phallus about 2.5 cm – Bifid scrotum with urethral opening like hypospadias	41	33	20.1	19	55	Free testosterone 1.3 ng/dl and DHT 22 ng/dl	No internal female genital organs, Rt scrotal testis and Lt inguinal one	45 X	+ ve	+ ve	45 X male syndrome
8	Abnormal urine stream	Genitalia shows – 2 palpable gonads – Phallus about 3.75 cm in stretched length – Bifid scrotum with urethral opening at base of phallus and scrotum	48	26	21	20	103		No internal female genital organs and bilateral labioscrotal gonads	46 XY	+ ve	+ ve	Penioscrotal hypospadias
9	Ambiguous genitalia	Genitalia shows – 2 palpable gonads – Phallus (3.2 cm) with longitudinal groove on ventral aspect – Urethral opening at base of phallus associated with bifid scrotum	55	44	19.8	18.7	70		No internal female genital organs and bilateral labioscrotal gonads	46 XY	+ ve	+ ve	Penioscrotal hypospadias
10	Ambiguous genitalia and respiratory distress	Genitalia shows – 2 palpable gonads – Phallus (3 cm) with longitudinal groove on ventral aspect – Urethral opening at base of phallus associated with bifid scrotum	52	28	17.4	12.5	89		No internal female genital organs and bilateral labioscrotal gonads	46 XY	+ ve	+ ve	Penioscrotal hypospadias
11	Ambiguous genitalia and abnormal urine stream	Genitalia shows – 2 palpable gonads – Phallus (1 cm) with longitudinal groove on ventral aspect – Urethral opening at base of phallus associated with bifid scrotum	39	34	22	19.2	94		No internal female genital organs and bilateral labioscrotal gonads	46 XY	–	–	Penioscrotal hypospadias and micropenis
12	Vomiting and dehydration attack	– Female like external genitalia – Shows 2 palpable gonads – Phallus (1.5 cm) with a single opening at its base on ventral aspect	178 ↑	88 ↑	2 ↓	2.2 ↓	17 ↓	DHEA 17.7 ng/dl ↓, androstendione 2.8 ng/dl ↓ and total testosterone 0.2 ng/dl ↓	No internal female genital organs and Rt inguinal testis and Lt labioscrotal one	46 XY	+ ve	+ ve	CAH
13	Ambiguous genitalia	Genitalia shows – Left palpable gonad, – Phallus (2 cm) – Urethral opening at base of phallus associated with bifid labioscrotal folds	30	22	11.3	13.3	75		No internal female genital organs and Rt inguinal, Lt labioscrotal gonads	46 XY	–	–	Penioscrotal hypospadias and micropenis

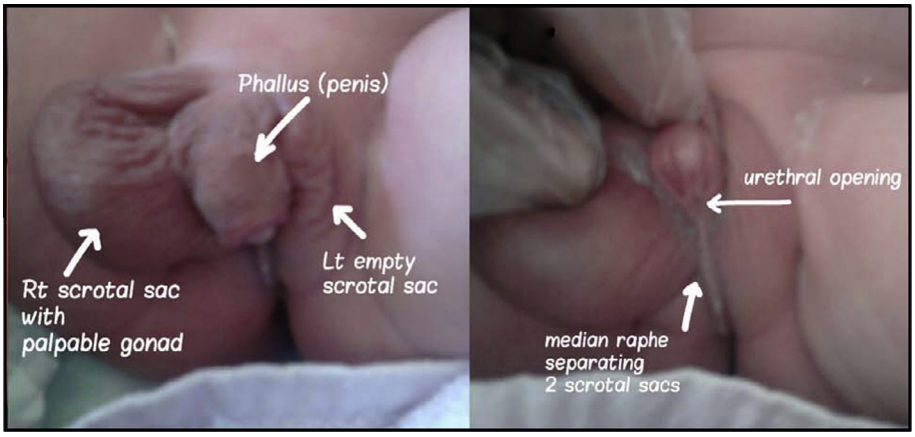


Figure 1 External genitalia of patient number (6).

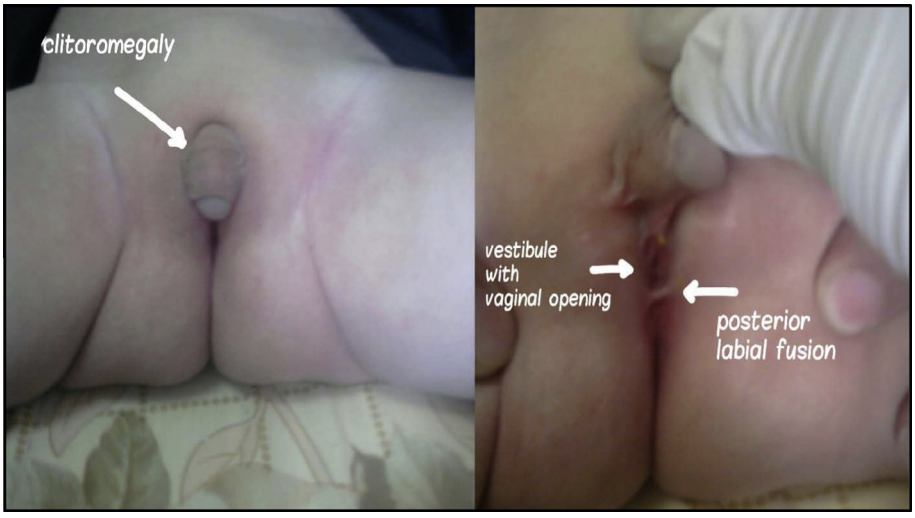


Figure 2 External genitalia of patient number (7).

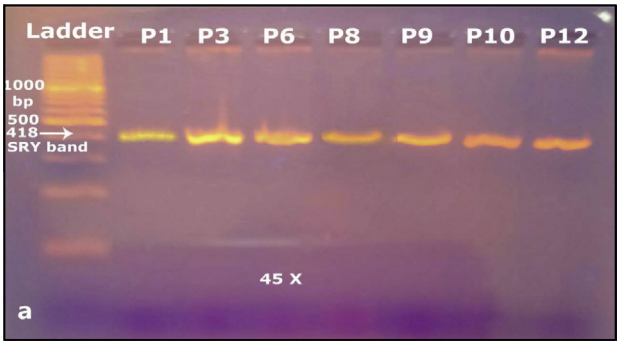


Figure 3 Gel electrophoresis of SRY gene in apparent male cases: First lane on the left shows 100 bp DNA ladder. SRY was present at position 418 bp in all clinically apparent male cases including case number (6) which had a karyotype of 45, X (with translocated SRY gene).

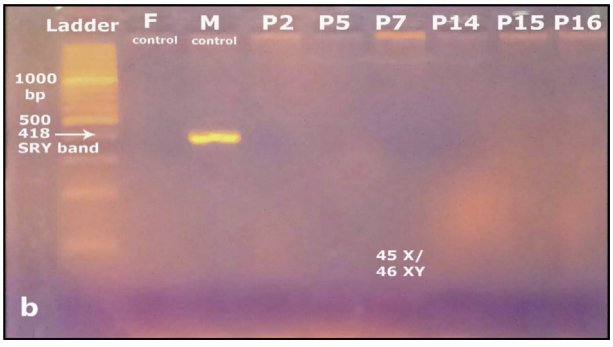


Figure 4 Gel electrophoresis of SRY gene in apparent female cases: First lane on the left shows 100 bp DNA ladder. F = female control and M = male control. Clinically apparent female cases showed no bands for SRY gene including case number (7) which had a karyotype of 45, X/46, XY(with deleted SRY gene).

As regards the anthropometric measurements, all patients were within normal ranges except in 4 cases which were below 3rd percentile for weight and < -2 SD for BMI, and only three of them were below 3rd percentile for length. This is consistent

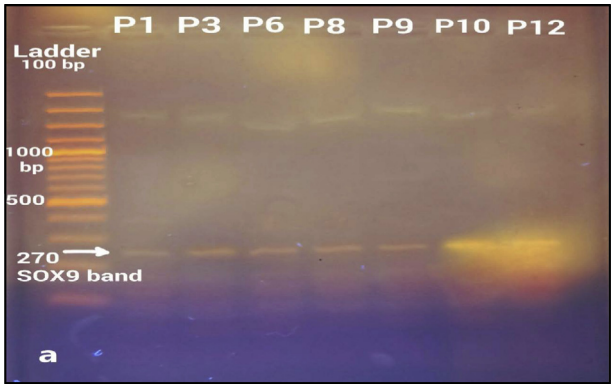


Figure 5 Gel electrophoresis SOX9 gene in apparent male cases: First lane on the left shows 100 bp DNA ladder. SOX9 was present at position 270 bp in all clinically apparent male cases.

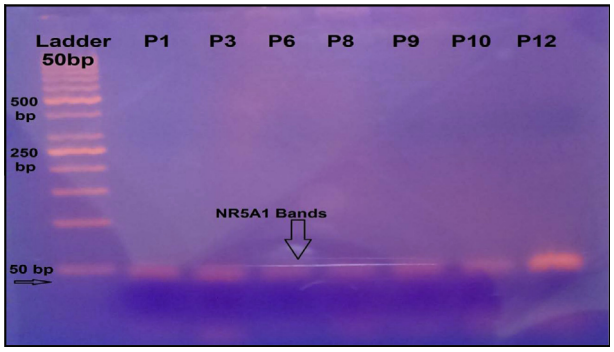


Figure 7 Gel electrophoresis of NR5A1 gene in apparent male cases: First lane on the left shows 50 bp DNA ladder. NR5A1 gene for apparent male cases was detected approximately at 50 bp position.

with the significance of serial anthropometric measurements as an important tool to insure that DSD especially salt losing CAH do not affect the child normal growth pattern and for follow up of treatment [19].

The external genital manifestations of the female patients were mainly clitoromegaly and fused labia, with no palpable gonads and two of them had abnormal pubic hair growth. Prader score for the apparent female patients in this study ranged from score 1 to score 4. These findings were consistent with the findings of Ogilvy-Stuart and Brain [20], who demonstrated that any apparent female case with DSD may present with a wide range of presentations.

For male patients, the external genital manifestations were mainly bifid scrotum and urethral opening at phallus base. Three cases had microphallus. Two had only one palpable gonad and one case looked apparently female (patient number 12). In addition, the external masculinization score (EMS), which is used to describe the degree of ambiguity in an undervirilized boy, was applied and all male cases scored below 11, which mean that they need to be evaluated carefully for possibility of DSD [8].

Tanner staging showed that all patients are in 1st pubertal stage except two female patients (4 and 16) who were with pubic hair stage 2 which was consistent with the early age of presentation and evaluation in majority of our cases depending on etiological nature of the disease. This demonstrated the dependency of Tanner stages on hormonal effects as regards secondary sexual characteristics [21].

Regarding the imaging studies, seven patients with apparent female phenotype had normal mullerian structures and gonads except case 7 who had bilateral pelvic streak gonads. Nine patients with apparent male phenotype showed normal inguinoscrotal gonads and no internal female genitalia. These findings were in line with Govind et al. [22] illustrations about imaging of ambiguous genitalia, and dependency of the results on the etiological nature of the disease.

Considering hormonal studies, we found that five patients were above normal ranges for serum 17 OH progesterone levels and two patients were below normal ranges. From these seven patients, six had elevated serum ACTH levels and low serum cortisol levels. In addition, five of them showed elevated serum adrenal androgen levels, and the other two showed decreased levels. This was consistent with the laboratory findings of CAH as demonstrated by Wajnrajch and New [23], indicating the cornerstone role of the measurement of these three hormones especially 17-OHP for establishing diagnosis of CAH.

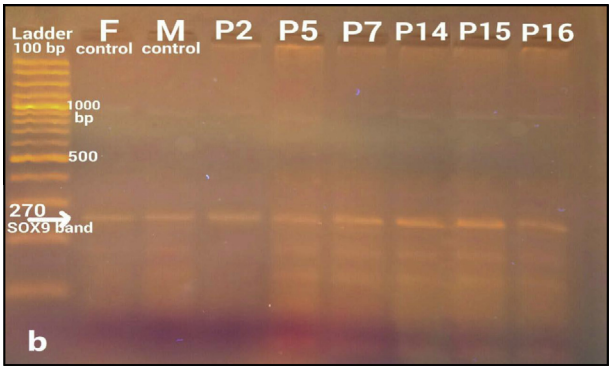


Figure 6 Gel electrophoresis of SOX9 gene in apparent female cases: First lane on the left shows 100 bp DNA ladder. F = female control and M = male control. SOX9 was present at position 270 bp in all clinically apparent male cases.

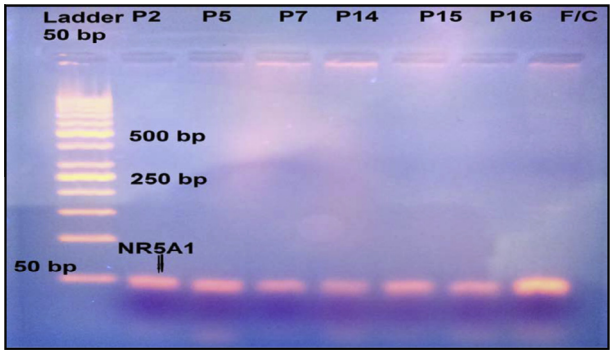


Figure 8 Gel electrophoresis of NR5A1 gene in apparent female cases: First lane on the left shows 50 bp DNA ladder. F/C = female control. NR5A1 gene for these cases was detected approximately at 50 bp position.

Four female patients (2,4,5,15) had elevated serum17 hydroxy progesterone level, low serum cortisol and high ACTH levels and elevated DHEA and androstendione levels, which is consistent with diagnosis of CAH due to 21 hydroxylase deficiency, the most common cause of CAH [15].

For patient number 1, we found mildly elevated serum17 hydroxy progesterone level, normal serum cortisol and ACTH levels and low DHEA and androstendione levels, which is consistent with diagnosis of CAH due to isolated 17,20-lyase deficiency [24].

Considering patient number 14, hormonal evaluation demonstrated low serum17-OHP level and cortisol levels, elevated serum ACTH levels, elevated serum DHEA level and low serum androstendione level, which is consistent with laboratory findings in cases with CAH due to 3-Hydroxysteroid Dehydrogenase deficiency [25].

Regarding patient number 12, hormonal evaluation showed decreased serum17-OHP, serum cortisol levels with elevated serum ACTH levels and low adrenal androgens levels. These findings were consistent with laboratory findings in cases with salt losing CAH due to 20-22 desmolase deficiency [26].

As regards other hormonal assays depending on the case, we found that patient number 3 showed high serum testosterone levels, low serum DHT level and high T:DHT ratio (37.4) after hCG stimulation test. Being with normal male karyotype, these findings were consistent with diagnosis of 5 α reductase deficiency [27].

The genetic study was divided into two main categories: karyotyping and molecular study. Karyotyping of the cases revealed six patients (37.5%) had normal female (46, XX) karyotype, eight patients (50%) with normal male (46, XY) karyotype and two (12.5%) with abnormal karyotypes (one with 45, X and another with 45, X/46, XY). This was consistent with Siklar et al. [28] who showed that 51.4% of cases with DSD had 46, XY karyotype, 34.9% with 46, XX and 13.7% with other conditions, and considering the limitations in our data collection may have led to estimation of less than the usual variation expressed by other authors. This also confirmed the essentiality of karyotyping for provisional diagnosis and classification of the DSD cases [10].

Regarding molecular study, we focused on three genes SRY, SOX9 and NR5A1 genes. Mutations or deletion of SRY gene can result in 46, XY sex reversal or gonadal dysgenesis [29] while translocation of Y chromosomal material including SRY gene can result in 46, XX male syndromes [30]. Deletion or mutations of SOX9 gene can result in a severe skeletal malformation syndrome (campomelic dysplasia) which is frequently associated with male to female sex reversal [31]. In humans, abnormalities in SF1 (NR5A1) gene can lead to adrenal and gonadal failure, severe penoscrotal hypospadias and sex reversal [32].

Regarding SRY gene, we detected SRY gene band at 418 bp position (Figs. 3 and 4) in all studied genetically male patients except patient number 7 which had a karyotype of 45, X/46, XY. Also for studied genetically female patients, no SRY gene bands were detected except patient number 6 which had a karyotype of 45, X. This is in line with role of SRY gene in sex determination towards a male line through the presence of Y chromosome or its translocation to another chromosome mostly X chromosome [30].

As regards SOX9 gene, the results demonstrated the presence of SOX9 gene band nearly at 270 bp position in all cases (Figs. 5 and 6). Therefore, no deletions for SOX9 gene were detected in our studied cases and this was in line with clinical manifestations of studied cases in which no single case showed skeletal malformation.

As regards NR5A1 gene, the results demonstrated the presence of NR5A1 gene band nearly at 50 bp position in all patients (Figs. 7 and 8). Therefore, no deletions for NR5A1 gene were detected in our studied patients. This may be due to small sample size and the presence of microdeletions and mutations that need more advanced techniques as gene sequencing.

Regarding patient number 6 who presented to us with ambiguous genitalia from the 1st day of life. Local examination showed bifid scrotum with only palpable right testis and phallus 2.5 cm in length with proximal hypospadias (Fig. 1). Imaging studies confirmed the presence of right scrotal testis, left inguinal gonad and absence of Mullerian structures. Hormonal evaluation was normal for adrenal and testicular functions. These results were consistent with being undermusculinized male, but cytogenetic studies including Fluorescent insitu hybridization revealed a case of 45, X. This directed evaluation toward the possibility of a translocated SRY gene, and the molecular study for SRY gene revealed its presence in this case (Fig. 3) as reported in other studies [33].

Patient number 7 was clinically an apparent female on external genitalia and stage 2 on Prader scale (Fig. 2). Laboratory evaluation was normal and imaging studies including MRI showed uterus and bilateral pelvic streak gonads. These results were consistent with being virilized female, but cytogenetic studies including FISH revealed a case of 45, X/46, XY. These findings were consistent with diagnosis of mixed gonadal dysgenesis [34].

The predominance of the X or XY cell lines determines the gonadal differentiation into a testis or a streak gonad [35] which was in line with this patient which had bilateral streak gonads and 7% male cell lines. In addition, the molecular study determined the absence of SRY gene (Fig. 4). This suggests that the deleted SRY gene played a role in maintaining female phenotype and Mullerian structures despite the presence of Y chromosome. The occurrence of mutations in the SRY gene was reported in patients with 45, X/46XY karyotype [36,37].

During this study, the main challenges were the parents of the studied cases, who showed marked irritability about their children's condition, the spectrum of DSD, which can pose a diagnostic dilemma and obtaining the material for this study. The parents were anxious to know their children's condition and to reach a diagnosis as soon as possible, which was met from our side with assurance and need of appropriate full anatomical, biochemical and multidisciplinary evaluation before rushing to an arbitrary gender assignment. It was really a challenge.

5. Conclusion

The birth of a child with ambiguous genitalia is a matter of a medical and social emergency. Careful examination of external genitalia with emphasis on gonadal detection, uniform classification of DSD, good cytogenetic and molecular facilities, individualized approach with integrated team management and supportive counseling form the mainstay of management of DSD. It is also important that appropriate counseling is available for the individual and family throughout their life and that the clinical and research communities work together to

determine the molecular basis of disorders of sex development when no changes in SOX9, NR5A1 or other candidate genes are found.

Conflict of interest

We have no conflict of interest to declare.

References

[1] Nikolova Ganka, Vilain Eric. Mechanisms of disease: transcription factors in sex determination relevance to human disorders of sex development. Nat Clin Pract Endocrinol Metab April 2006;2:231–8.

[2] Lin L, Achermann JC. Steroidogenic factor-1 (SF-1, Ad4BP, NR5A1) and disorders of testis development. Sex Dev 2008;2:200–9.

[3] Buaas FW, Gardiner JR, Clayton S, Val P, Swain A. In vivo evidence for the crucial role of SF1 in steroid-producing cells of the testis, ovary and adrenal gland. Development 2012;139:4561–70.

[4] Büdefeld T, Tobet SA, Majdic G. Steroidogenic factor 1 and the central nervous system. J Neuroendocrinol 2012;24:225–35.

[5] Cohen-Bendahan CC, van de Beek C, Berenbaum SA. Prenatal sex hormone effects on child and adult sex-typed behavior: methods and findings. Neurosci Biobehav Rev 2005;29:353–84.

[6] Kim Kun Suk, Kim Jongwon. Disorders of sex development. Korean J Urol 2012;53(1):1–8.

[7] Prader A. Genital findings in the female pseudohermaphroditism of the congenital adrenogenital syndrome; morphology, frequency, development and heredity of the different genital forms. Helv Paediatr Acta 1954;9(3):231–48.

[8] Ahmed SF, Rodie M. Investigation and initial management of ambiguous genitalia. Best Pract Res Clin Endocrinol Metab 2010;24:197–218.

[9] Tanner. J Pediatr 1985;107(3):317.

[10] Lee PA, Houk CP, Ahmed SF, Hughes IAInternational consensus conference on intersex organized by the Lawson Wilkins pediatric Endocrine Society and the European Society for paediatric endocrinology. Consensus statement on management of intersex disorders. International consensus conference on intersex. Pediatrics 2006;118:e488–500.

[11] Speiser PW, White PC. Congenital adrenal hyperplasia. N Engl J Med 2003;349:776–88.

[12] Houk CP, Lee PA. Consensus statement on terminology and management: disorders of sex development. Sex Dev 2008;2:172–80.

[13] Barch MJ, Knutsen T, Spurbeck JL. The AGT cytogenetics laboratory manual. Philadelphia: Lippincott-Raven; 1997, vol. xiii, p. 666.

[14] Hughes IA. Disorders of sexual differentiation. Horm Res 2007;67(Suppl. 1):91–5.

[15] Clayton PE, Miller WL, Oberfield SE, Ritzén EM, Sippell WG, Speiser PW. Consensus statement on 21-hydroxylase deficiency from the European Society for paediatric endocrinology and the Lawson Wilkins pediatric Endocrine Society. Horm Res 2002;58:188–95.

[16] Mazen I, Hiort O, Bassiouny R, El-Gammal M. Differential diagnosis of disorders of sex development in Egypt. Horm Res 2008;70:118–23.

[17] Nimkarn S, Lin-Su K, New MI. Steroid 21 hydroxylase deficiency congenital adrenal hyperplasia. Pediatr Clin North Am 2011;58:1281–300.

[18] Warne GL, Raza J. Disorders of sex development (DSDs), their presentation and management in different cultures. Rev Endocr Metab Disord 2008;9:227–36.

[19] Dauber A, Kellogg M, Majzoub JA. Monitoring of therapy in congenital adrenal hyperplasia. Clin Chem 2010;56(8):1245–51.

[20] Ogilvy-Stuart AL, Brain CE. Early assessment of ambiguous genitalia. Arch Dis Child 2004;89(5):401–7.

[21] Traggiai C, Stanhope R. Disorders of pubertal development. Best Pract Res Clin Obstet Gynaecol 2003;17(1):41–56.

[22] Govind BC, Dimitri AP, Kamaldine O, et al. Imaging of ambiguous genitalia: classification and diagnostic approach. Radio Graph 2008;28:1891–904.

[23] Wajnrajch MP, New MI. Defects of adrenal steroidogenesis. In: Jameson JL, DeGroot LJ, editors. Endocrinology. Adult and Pediatric. Philadelphia, PA: Saunders Elsevier; 2010. p. 1897–920.

[24] Geller DH, Auchus RJ, Mendonca BB, Miller WL. The genetic and functional basis of isolated 17,20-lyase deficiency. Nat Genet 1997;17:201–5.

[25] Speiser PW, Azziz R, Baskin LS, Ghizzoni L, Hensle TW, Merke DP. Congenital adrenal hyperplasia due to steroid 21-hydroxylase deficiency: an Endocrine Society clinical practice guideline. J Clin Endocrinol Metab Sep 2010;95(9):4133–60.

[26] Bhangoo A, Anhalt H, Ten S, et al. Phenotypic variations in lipoid congenital adrenal hyperplasia. Pediatr Endocrinol Rev 2006 Mar;3(3):258–71.

[27] Imperato-McGinley J, Zhu YS. Androgens and male physiology the syndrome of 5alpha-reductase-2 deficiency. Mol Cell Endocrinol 2002;198(1–2):51–9.

[28] Siklar Z, Berberoglu M, Adiyaman P, Salih M, Tukun A, Cetinkaya E, et al. Disorders of gonadal development: a broad clinical, cytogenetic and histopathologic spectrum. Pediatr Endocrinol Rev 2007;4:210–7.

[29] Bashamboo A, McElreavey K. Gene mutations associated with anomalies of human gonad formation. Sex Dev 2013;7:126–46.

[30] Huang WJ, Yen PH. Genetics of spermatogenic failure. Sex Dev 2008;2:251–9.

[31] Benko S, Gordon CT, Mallet D, et al. Disruption of a long distance regulatory region upstream of SOX9 in isolated disorders of sex development. J Med Genet 2011;48:825–30.

[32] Köhler B, Achermann JC. Update – steroidogenic factor 1 (SF-1, NR5A1). Minerva Endocrinol 2010;35:73–86.

[33] Wimmer R, Schempp W, Gopinath PM, et al. A family case of fertile human 45, X, psu dic (15;Y) males. Cytogenet Genome Res 2006;115(1):94–8.

[34] Telvi L, Lebbar A, Del Pino O, Barbet JP, Chaussain JL. 45, X/46, XY mosaicism: report of 27 cases. Pediatrics 1999;104:304–8.

[35] Rimoin DL, Connor JM, Pyeritz RE, Korf BR. Emery and Rimoin's principles and practice of medical genetics. 5th ed. Philadelphia, USA: Churchill Livingstone; 2006.

[36] Shahid M, Dhillon VS, Aslam M, Husain SA. Three new novel point mutations localized within and downstream of high-mobility group-box region in SRY gene in three Indian females with turner syndrome. J Clin Endocrinol Metab 2005;90:2429–35.

[37] Dumic M, Lin-Su K, Leibel NI, et al. Report of fertility in a woman with a predominantly 46, XY karyotype in a family with multiple disorders of sexual development. J Clin Endocrinol Metab 2008;93:182–9.