# CYTOGENETIC AND MOLECULAR ANALYSIS OF PATIENTS WITH AMBIGUOUS GENITALIA

**Shatha R.Zaidan, Abdul Hussain Moyet Al-Faisal & Nahia A. Ali**

## ABSTRACT

**Ambiguity is one of the sex determination disorders which are caused by several genetic and hormonal factors. This study was carried out to determine the association between sex external genitalia and genetic phenotype and to find the role of SRY in ambiguity. Twenty one patients with ambiguous genitalia were involved in the study. Blood samples were used for cytogenetic analysis and for DNA extraction for PCR analysis with SRY and SMCX primers.In this study we examined 21 patients with ambigiuity and we grouped them into two groups according to their external genitalia. The first group consists of 6 patients with apparently female external genitalia and the second group consists of 15 patients with apparently female external genitalia plus micropenis. Cytogenetic and molecular analyses were preformed and the cytogentic analysis revealed that 4 patients of group-1 were with 46,XY karyotype and two with 46,XX karyotype. While 14 patients of group-2 were with 46,XX and one with mosaic karyotype. PCR analysis of the DNA extracted from patients blood of both groups with SRY and SMCX primers or with SRY primers alone showed that discordance between PCR and cytogenetic results was detected in patient no.5 from group-1 and patients no.7 and 8 from group-2 where the PCR positive to SRY while the karyotype is 46,XX. Discordance was detected between the external genitalia and cytogenetic karyotype of the patients with ambiguity. Such discordance was confirmed by PCR analysis.**

## INTRODUCTION

Ambiguous genitalia is a rare disorder which caused by defects in the process of fetal sexual determination and differentiation. Sex determination which depends on sex-chromosome complement, governed and regulated by different genetic keys which role the cellular differentiation during critical periods of gonadal development.[1-3] Gonads are bipotential and the maleness of fetus depends on the Y chromosome and is linked to sex-determining region of chromosome Y (SRY).[4] In the absence of SRY, the fetus will develop female internal and external genitalia. In the presence of SRY, cells of the gonads become testicular Sertoli cells and without SRY, the supporting cells become ovarian follicular cells. Sex determination sets the stage for sex differentiation, where is the sexual tissue response specifically to hormones produced by gonads and the external genitalia differentiated toward the male phenotype in the presence of testosterone and dihydrotestosterone and toward the female phenotype in the presence of Mullerian substance hormone.[4-6] If these processes are disrupted ambiguous genitalia can be developed. The extent of the ambiguity varies and in many cases the phenotype appearance may be fully or partially developed as the opposite of the genetic sex. These disorders have been shown to be associated with different genetic, endocrinological and environmental factors.[2,7] Many types of genetic abnormalities such as female 46,XY or male 46,XX have been reported.[7-9] Ambiguity results from a partial form of XY gonadal dysgenesis or from mosaicism such as 45,XO/46XY or 46,XX/46,XY were also reported.[5,10] Mutations, deletions and translocations of SRY or other genes important to the external genitalia developments were also well documented.[3] Many types of hormones deficiencies which were associated with some ambiguous categories such as ambiguity with micropenis were detected such as 21-hydroxylase deficiency,[11-13] 11-hydroxylase deficiency and 3 beta-hydroxysteroid dehydrogenase deficiency. [14] In this work, we investigated the cytogenetic status and the role of the gene SRY in two categories of ambiguity, patients with normal appearing external female genitalia and patients with female genitalia plus micropenis or full penis.

**Shatha R. Zaidan, MSc, Abdul Hussain Moyet Al-Faisal, PhD and Nahia A. Ali, PhD***
***Institute of Genetic Engineering and Biotechnology/Baghdad University/Iraq**
**Abdul Hussain M.Al-Faisal: alfais2000@yahoo.com**

## MATERIALS & METHODS

***Grouping of the Ambiguity Patients:***

Patients were grouped into two groups based on the phenotype of the external genitalia:

***Group-1*** which included 6 patients with apparently female genitalia (patients No. 1, 2, 3, 4, 5 and 6).

***Group-2*** which included 15 patients with apparently female genitalia plus micropenis or full penis (patients No. 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20 and 21) ( Figure-1 ).

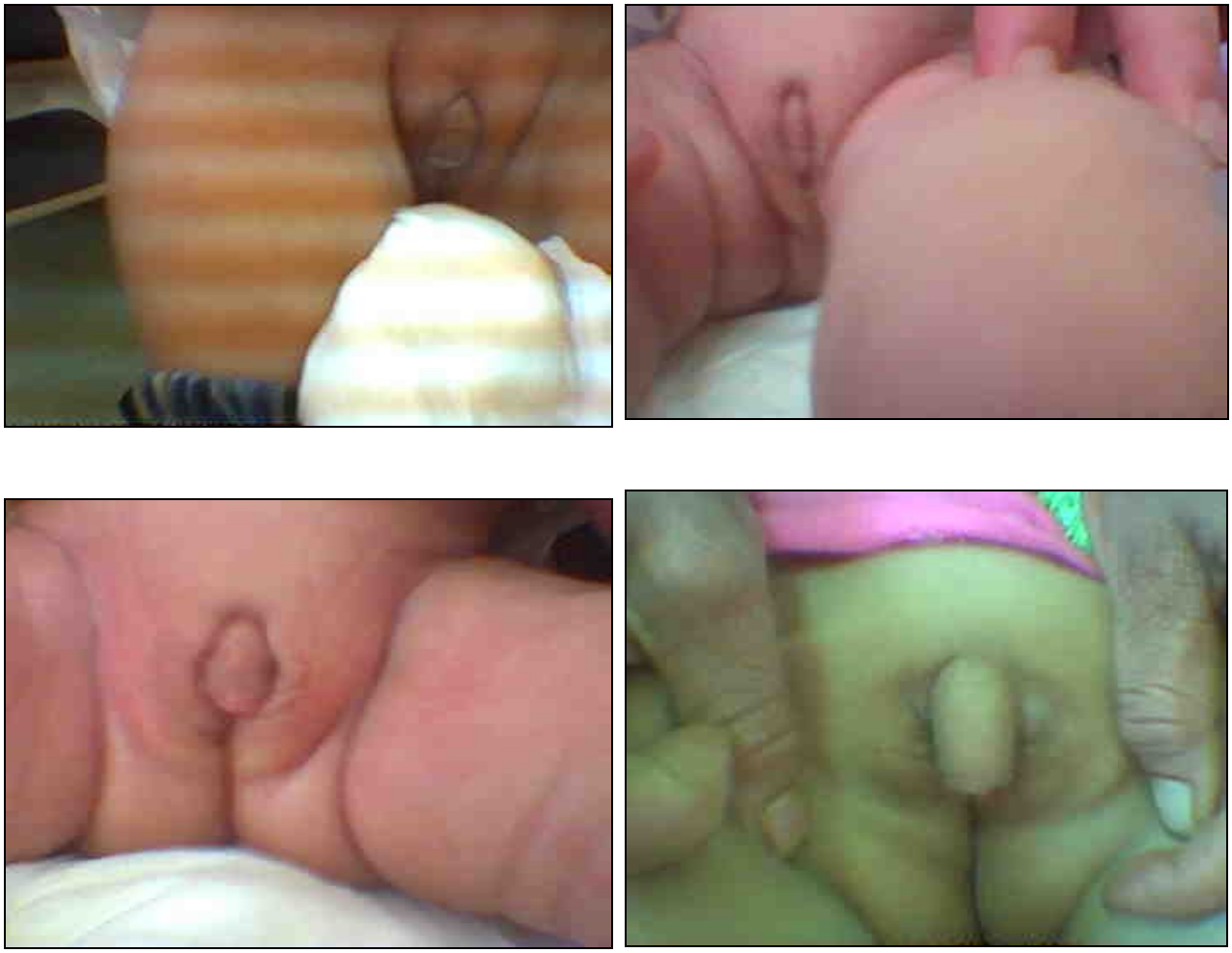

***Fig 1. Different external genitalia abnormalities distributed among ambiguity patients included in this study.***

***Blood Sampling:***

Five ml of blood was collected by veni puncture from 21 patients (patient ages ranging from 1 month to 18 years) with ambiguity who were referred to Medical City/Baghdad from October 2006 to may 2007 with a disposable syringe. Each blood sample was divided into two aliquots, one aliquot was added to heparinized tube for cytogenetic examination, and the other aliquot was added to EDTA tube for DNA extraction. The EDTA blood samples were centrifuged at 2000 rpm for 10 minutes. The serum of each blood sample was collected in a clean and sterile tube and used for hormonal assays. The WBC layer from each sample was collected in a sterile tube and used in DNA extraction.

***Blood Culture and Slides Preparation:***

Blood culture and slides preparation were performed according to Jyothy et al, 2002[8]. 0.5 ml from each heprinized blood sample was cultured in 5 ml of standard supplemented RPMI 1640 medium containing 20% fetal calf serum and 2% of phytohemagglutinin (PHA) (prepared by the molecular biology Department, Iraqi center for cancer and medical genetic research-Baghdad-Iraq) in a sterile tubes. The tubes were cultured at 37ºC for 72 hours.100 microliter of cholchicine (0.45 mg\ml) was added to each culture. After 20 minutes, the cells from all culture tubes were harvested by centrifugation (2000rpm\10 mins). The supernatants were discarded and the cells redissolved with the remaining solution. The cells were exposed to mild hypotonic treatment with 3ml of 0.075 M KCL at 4C. The cells were precipitated by another centrifugation. The supernatants were discarded, cells resuspended with remaining hypotonic solution and fixed with 5 ml fixative solution (3 methanol:1 Glacial acetic acid). Centrifugation and fixation were repeated four times at intervals of 20 minutes. Then, the slides were treated with trypsin for 20-30 second, stained for 10 minutes with 5% buffered Giemsa solution, pH 6.8. Three slides were prepared for each sample and 50 metaphases were examined from each sample for chromosomal abnormalities under light microscope.

***DNA extraction:***

The WBC layers which were collected from the EDTA blood samples were used in DNA extraction. The DNA was extracted according to the Wizard genomic DNA purification kit (Progema/USA).0.3-ml from the WBC suspension was mixed with 900 microliter of cell lysis buffer. Samples were incubated at 20C for 10 minutes. The nuclei were pelleted by centrifuging at 3000 rpm for 10 minutes. The supernatant was discarded and the pellet redissolved with the remaining solution.300 microliter from nuclei lysis buffer was added to the nuclei suspension with gentle mixing for one minute then 300 microliter from protein lysis solution was added with another mixing. The samples were then centrifuged, the supernatants were collected in a clean tubes and the DNA precipitated with equal volume of isopropanol alcohol. DNA samples were pelleted by centrifugation, washed with 70% ethanol alcohol, air dried and resuspended with 100 microliter of distilled water. The DNA concentration and purity were checked. The agarose gel electrophoresis was also adopted to confirm the presence and integrity of the extracted DNA.

***PCR Assay:***

PCR reaction was performed using the SRY and SMCX specific primers. Lyophilized primers were dissolved in a sterile distilled water to give a final concentration of 10p mol/µl. The sequences of these primers were listed in Figure-2.

| STS | Forward | Reverse | Molecular size |
|---|---|---|---|
| SRY | 5-GAATATTCCGCTCTCCGGA-3 | 5-GCTGGTGCTCCATTCTTCAG-3 | 472 bp |

| STS | Forward | Reverse | Molecular size |
|---|---|---|---|
| SMCX | CCGCTGCCAAATTCTTTGG | TGAAGCTTTTGGCTTTGAG | 310 bp |

***Fig 2. Primers sequences and products.***

PCR was performed according to Saiki et al, 1988(15) using a thermal DNA cycler machine (Techgene-UK). Cinagene PCR Kit (Iran) was utilized. 100 ng of denaturated DNA and 40 picomole from each primers were added to the PCR master mixture. The reaction was initiated in a volume of 50 ul. A total of 20 cycles of polymerization was carried out (Table-1). 10 ul from each amplified DNA , 0.2 ug of lambda Hind III+EcoR1 fragments as a marker were mixed with 2 ul of loading buffer and electrophoresed through a 1% agarose gel for 30 minutes at 50 Hz volts. The gel was then stained, visualized under UV light and photographed.

***Table 1. The PCR reaction conditions.***

| PCR with SRY primers | | | | |
|---|---|---|---|---|
| **No.** | **Steps** | **Temperature** | **Time** | **No. of cycles** |
| **1.** | Denaturation 1 first loop | 95 C° | 3 min | 1 cycle |
| **2.** | Denaturation 2 | 94 C° | 1 min | 35 cycle |
| **3.** | Annealing | 55 C° | 1 min | |
| **4.** | Extension 1 | 72 C° | 1 min | |
| **5.** | Extension 2 | 72 C° | 5 min | 1 cycle |
| **PCR with SMCX primers** | | | | |
| **No.** | **Steps** | **Temperature** | **Time** | **No. of cycles** |
| **1.** | Denaturation 1 first loop | 95 C° | 3 min | 1 cycle |
| **2.** | Denaturation 2 | 95 C° | 1 min | 35 cycle |
| **3.** | Annealing | 58 C° | 1 min | |
| **4.** | Extension 1 | 72 C° | 1 min | |
| **5.** | Extension 2 | 72 C° | 10 min | 1 cycle |

## RESULTS

### *Cytogenetic Analysis:*

The cytogenetic analysis was focused on the assignment of patients whether they are males or females according to the sex chromosomes. The results showed some complexity (Table-2).

***Table 2. The Cytogenetic Results of the Ambiguous Patients Groups.***

| Groups | Patients No. | | | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| 1 | 1 | 2 | 3 | 4 | 5 | 6 | | | | | | | | | | No. of ♀ =2 |
| | XY | XY | XY | XY | XX | XX | | | | | | | | | | No. of ♀ =4 |
| 2 | 7 | 8 | 9 | 10 | 11 | 12 | 13 | 14 | 15 | 16 | 17 | 18 | 19 | 20 | 21 | No. of ♀ =14 |
| | XX | XX | XX | XX | XX | XX | XX | XX | XX | M | XX | XX | XX | XX | XX | No. of M=1 |

**M: Mosaic ⚥ : Female ♀ : Male**

Four cases have a karyotyp of 46,XY and two 46,XX karyotypes were revealed with patients of (Group-1) where the external genitalia of them were with predominant female genitalia. Fourteen cases of 46,XX and one case with 46,XX/46,XY karyotypes (mosaic) were revealed with patients of (Group-2) where the external genitalia of them with apparently female genitalia with a micropenis or fully penis. Also the cytogenetic analysis of all patients showed normal sex chromosomes without any type of abnormalities.

### *Molecular Analysis of Ambiguous Groups:*

The Genotype out come of groups patients were shown in Table-3.

***Table 3. Cytogenetic analysis and PCR with SRY of the ambiguous genitalia groups.***

| Groups | Patients No. | | | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| 1 | | 1 | 2 | 3 | 4 | 5 | 6 | | | | | | | | | |
| | cyto | XY | XY | XY | XY | XX | XX | | | | | | | | | |
| | SRY | + | + | + | + | + | - | | | | | | | | | |
| 2 | | 7 | 8 | 9 | 10 | 11 | 12 | 13 | 14 | 15 | 16 | 17 | 18 | 19 | 20 | 21 |
| | cyto | XX | XX | XX | XX | XX | XX | XX | XX | XX | M | XX | XX | XX | XX | XX |
| | SRY | + | + | - | - | - | - | - | - | - | + | - | - | - | - | - |

The results revealed that patients No. 1,2,3,4 and 6 were identical to their cytogenetic analysis. On other hand, discordance between PCR and cytogenetic results were detected with the patient No. 5. PCR analysis of patient No. 5 was corroborated the presence of SRY while the karyotype picture was 46, XX. Such results were also detected in patients No.7 and 8 from group-2.

In addition to the 46,XX positive to SRY identified in groups-1 and 2, mosaic positive to SRY (patient No.16) and 46,XX negative to SRY were also detected ( Figure 3 & 4 ).

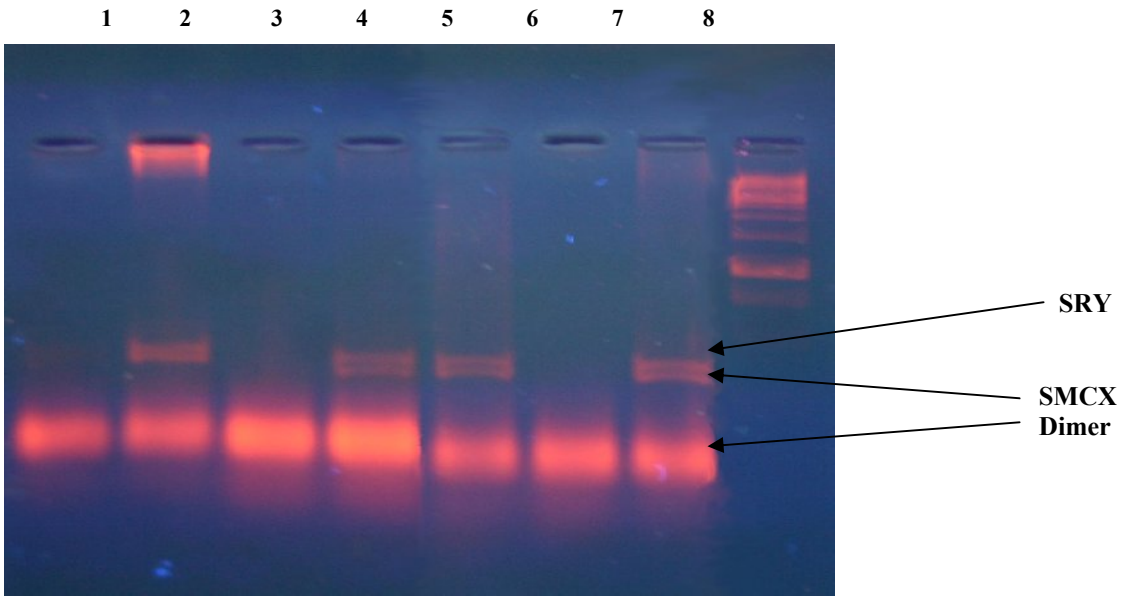


**Fig 3. PCR electrophoresis on agarose gel (2%) 45 min/70 volt.**
***Line 1+3+6: PCR of female samples with SRY primers (no bands).***
***Line 2+4+5+7: PCR of male samples with SRY+SMCX primers.***
***Line 8: DNA marker fragments***

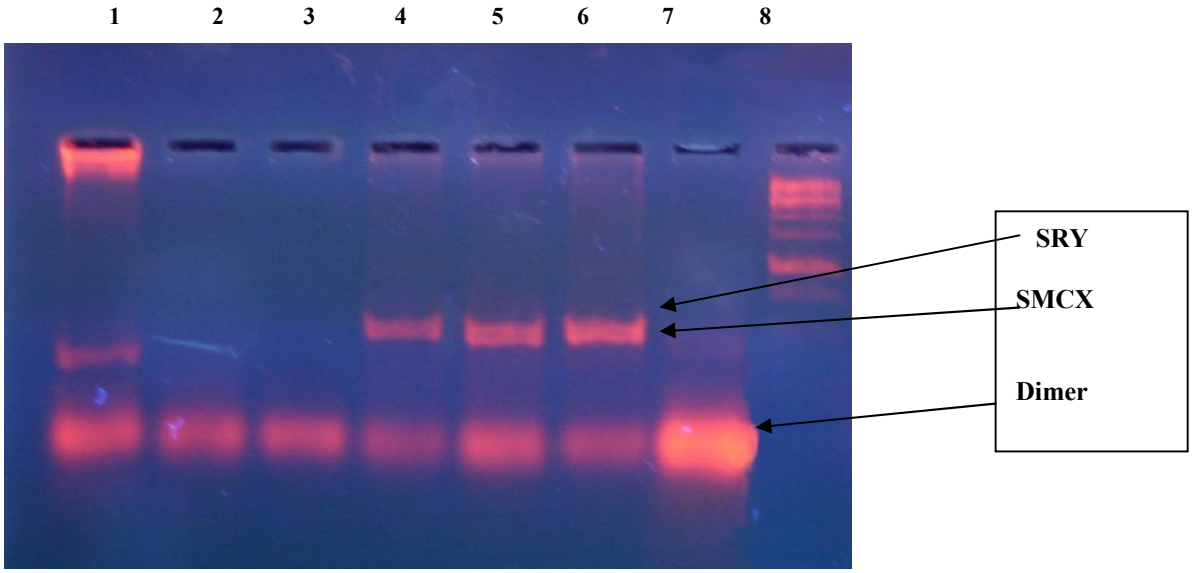


**Fig 4. PCR electrophoresis on agarose gel (2%) 45 min/70 volt.**
***Line 1+4+5+6: PCR of males with primers (SRY, SMCX).***
***Line 2+3+7: PCR of females with primer (SRY).***
***Line 8: DNA marker fragments.***

## DISSCUSION

The complexity revealed by the cytogenetic analysis reflects the complexity of the ambiguity. Normal sexual development requires the compatibility between genetic sex, phenotypic sex and gonadal sex.[16] Disorder in any of these can cause disorders of sexual development. The discordant phenotype/sex chromosomal patterns which were detected in this study reflect the complexity of the ambiguity. Although the etiology and complexity of these disorders is not clearly known, many types of these disorders were found to be caused by different genetic, endocrinological and environmental factors and many suggestions have been put forward to explain these disorders.[13] One of these suggestions is that genes other than SRY located on the Y chromosome are important for complete male gonadal differentiation. Such genes have been proved to have a role in sex differentiation[3]. One of these genes is the anti-testis development DAX1 gene which is located on the short arm of the chromosome X (Xp21.3-21.1) and a member of the nuclear hormones receptor family.[8] DAX1 gene is a part of large locus termed Dosage Sensitive Sex Reversal (DSS) located on Xp21 that may contain a wolffian inhibitory gene to suppress male differention.[4] This gene can alter SRY activity during development by suppressing downstream genes that induce testis differentiation and the female 46,XY can be arise after direct inhibition of SRY activity.[17,18] Also female 46,XY can be arose by indirect inhibition of SRY gene through effecting the SRY ability to up regulate SOX9 and antimullerian hormone genes expression.[19,20] WNT4 gene which is located on the short arm of the chromosome 1 (1p34 ) is also implicated in sex reversal and though to serve as anti-testis gene during male development.[4] Deletion of WNT4 gene has been associated with XY female sex reversal in patients with 1p31-35 duplication.[4] SF1 and WT1 have also been recognized for their role in the differentiation of bipotential gonads into either testis or ovaries.[5,19] Also the findings of this study, females 46,XX who are positive to SRY supported the influence of autosomal or X-linked genes in sex determination. Other researchers proposed other suggestions to explain the sex differentiation disorders. Jyothy et al, 2002[8] proposed that these disorders caused by sporadic errors in meiosis that produce chromosomal abnormalities by sending a confused or aberrant message to the process of gonadal differentiation. Such these chromosomal abnormalities associated with ambiguity were well documented.[2,9,10,13,21-24] Other explanations of sex disorders include exchange in the paternal germ line of terminal portion of Xp and Yp including SRY gene[7], a total lack of Leydig cells and thus a complete inability to produce testicular androgens results in external genitalia with a normal female appearance or due to imbalance between the production of androgens and Mullerian inhibitory substance (MIS)[20], hidden mosaicism[2,7], loss of function mutation in a gene participating in the sex-determining cascade[25] and a defect on a yet unidentified autosomal or X-linked sex determining genes.[26,27] Among 15 patients from group-2, 12 females showed a negative PCR results with SRY which indicated that the micropenis could be due to a kind of hormonal musculinization. Such cases were also detected by other studies.[4,12,13,28] They overmusculinized genitalia in females 46, XX is most probably caused by Congenital adrenal hyperplasia (CAH) resulting from 21-hydroxylase deficiency[29], 11-hydroxylase or 3 bata hydroxysteroid deficiencies.[14]

Finally, our result showed there is no direct role to SRY gene in ambiguity of genitalia but may play indirect role via cooperation with other genes.

## REFERENCES


1. **Donahoe DT, Maclaughlin, PK. Sex determination and differentiation. New England Journal of Medicine. 2004; l 350: 367-378.**
2. **Temel SG, Gulten, T, Yakut T, Saglam H, Kilic N, Bausch E, et al. Extended pedigree with multiple cases of XX sex reversal in the absence of SRY and of a mutation at the SOX9 locus. Sexual Development. (2007); 1: 24-34.**
3. **Wilhelm D, Palmer S, Koopman P. Sex determination and gonadal development in mammals. Physiol Reviews, 2007; 87(1): 1-28.**
4. **Hughes IA. Intersex. Brit.J.Urol.Internal. 2002; 90:769-776.**

5. Maclauglin DT, Donahoe PK. Sex determination and differentiation. The New England J. Medicine, 2004; 350(4): 367-378.
6. Wilson JD. The role of androgens in male gender role behavior. Transgender. 2006; 50: 44-70.
7. Ellaithi M, Gisselsson D, Nilsson T, Abd El-Fatah S, Ali, T, Elagib A, Ibrahim M, E, Fadl-Elmula I. A del (X) (p11) carrying SRY sequences in an infant with ambiguous genitalia. BMC Pediatrics, 2006*; 6:* 11.
8. Jyothy A, Kumar KSD, Swarna M, Raja Sekhar M, Uma Devi B, Reddy P P.. Cytogenetic investigations in 1843 referral cases of disordered sexual development from Andhra Pradesh, India. IGHG, 2002; 2 (1):55-59.
9. Ma S, Tang SS, Yuen B Ho, Bruyere H, Penaherrera M, Robinson W,P. Cytogenetic and molecular study of a premature male infant with 46, XX derived from ICSI. Human Reproduction, 2003; 18(11): 2298-2301.
10. Hughes IA, Houk C, Ahmed SF, Lee PA. LWPES/ESPE Consensus Group: Consensus statement on management of intersex disorders. Arch Dis Child, 2006; 91: 554-563.
11. Jimenez AL, Kofman,-Alfaro S, Berumen J, Hernandez E, Canto E. Partially deleted SRY gene confined to testicular tissue in an 46, XX true hermaphrodite without SRY in leukocytic DNA. Am J Med Genet. 2000; 93: 417-420.
12. Berenbaum SA. Cognitive function in congenital adrenal hyperplasia. Endocrinology and metabolism clinics of North America, 2001*; 30*: 173-192.
13. Migeon CJ, Wisniewski AB, Brown TR. et al. 46, XY intersex subjects: phenotypic and etiologic classification, knowledge of condition and satisfaction with knowledge in adulthood. Jounal of Paediatrics, 2002; 110: 32.
14. Schnitzer JJ, Donahoe PK. Surgical treatment of congenital adrenal hyperplasia. Endocrinol Metab Clin North Am; 2001; 30: 137-154.
15. Saiki RK. The design and optimization of the PCR. In: "PCR Technology Principles and Applications for DNA amplification" edt. Erlich, H.A. Stockton Press, New York, 1988.
16. Forest MG. Etiopathogenesis, classification, investigation and diagnosis in intersex. Indian J Pedial, 1992; 59: 475-485.
17. Fleming A, Vilain E. The endless quest for sex-determining genes. Clin Genet.2004; 67: 15-25.
18. Pop R, Conz C, Lindenberg KS, Blesson S, Schmalenberger B. Screening of the 1 Mb SOX95' control region by array CGH identifies a large deletion in a case of campomelic dysplasia with XY sex reversal. J Med Genet. . 2004; 41: 47.
19. Mendonca BB, Domenice S, Costa EMF, Arnhold IJP. Male pseudohermaphroditism. In New M (ed.) paediatric Endocrinology. 2002; 5: 20-25.
20. Migeon CJ, Wesniewskin AB. Human sex differentiation and its abnormalities. Best Practice and Research clinical abstract and Gynaecology. 2003; 17, pp.1-18.
21. Sarafoglou K, Ostrer H. Familial sex reversal: a review. J Clin Endocrinol Metab.2000; 85(2): 483-493.
22. Misra M, Maclaughlin DT, Donahoe PK, Lee MM. Measurement of mullerian inhibiting substance facilitates management of boys with microphallus and cryptorchidism.J.Clin. Endocrinol. Metab., 2002; 87(8): 3598-3602.
23. Shahid M, Dhillon VS, Jain N, Hedau S, Diwakar S, Sachdeva P, Batra S, Das BC, Husain SA. Two new novel point mutations localized upstream and downstream of the HMG box region of the SRY gene in three Indian 46,XY females with sex reversal and gonadal tumour formation. Molecular Human Rep. 2004; 10, pp, 521-526.
24. Zenteno JC,Lopez M,Vera C,E,T,A,L. Two SRY negative male brothers without genital ambiguity. Hum.genet. 1997; 100:606-610.
25. Vernole P, Terrinoni A, Didona B, et al. An SRY-negative XX male with Huriez syndrome. Clin. Genet, 2000; 57: 61-67.
26. Abu-Sheikha N, Lass A, Brinsden P. XX males without SRY gene and with infertility. Human Reproduction, 2001*; 16, 4*: 717-718.
27. Meyer-Bahlburg HFL. Gender and sexuality in classic congenital adrenal hyperplasia. Endocrinology and metabolism clinics of North America, 2001; 30: 155-171.
28. Migeon CJ, Wisniewski AB. Congenital adrenal hyperplasia owing to 21-hydroxylase deficiency: growth, development and therapeutic considerations. Endocrinology and metabolism clinics of North America, 2001; 30: 193-206.