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# Biochemical, Cytogenetic and Molecular Evaluation of Ambiguous Genitalia in Pediatric Patients

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## ABSTRACT

**Objective-** Ambiguity in genitalia is a rare phenotypic presentation of genitourinary system which needs immediate attention to evaluate the life-threatening disorder CAH (Congenital Adrenal Hyperplasia) and sex assignment. Hence determining the underlying etiology as soon as ambiguity is observed holds significant importance. The aim of the current study was to investigate the genesis and characteristics of ambiguous genitalia concerning the newborn.

**Materials and methods-** Biochemical, cytogenetic and molecular assessment was carried out in this study. A total of 100 cases were evaluated for CAH by biochemical analysis. GTG Karyotyping was done for cytogenetic analysis. The patients were further evaluated for Y chromosomal loci (SRY) with Polymerase Chain Reaction. PCR and sequencing based assay were further carried out in one of the cases with CAH for CYP21A2 gene mutation analysis.

**Result –** A total of 100 cases were successfully screened. Biochemical testing for 17- OH progesterone was done and 4 patients were found positive for CAH. On cytogenetic analysis, out of 100 cases, 4cases (4%) demonstrated mosaicism, rest 96 cases (96%) demonstrated a 46, XX or 46, XY karyotype. 6 cases (6%) carried a Y chromosome (46, XY) but were phenotypically female and 5 cases (5%) depicted female karyotype (46, XX) but were phenotypically male. Out of the 11 sex reversed cases, 7 were found positive for SRY gene.

**Conclusion-** In cases of children with suspected chromosomal anomalies to reveal the contribution of chromosomal disorders in the genesis of sexual ambiguity, molecular cytogenetic assessment can provide valuable insights to solve the problem of sexual ambiguity so that the problem can be resolved as soon as possible to enable effective genetic counseling.

***Keywords:*** Ambiguous genitalia, SRY gene, karyotype, CAH

## INTRODUCTION

The most common question which arises with birth of a child is about its gender. Whether the baby is male or female, the gender affects almost every aspect of their life. However, the profound differences among females and males, and the mechanisms by which such differences arise, are seemingly simple questions but with complex mechanisms. Ambiguity in sexuality is a complex and usually a confusing medical problem in newborn. More than 80% of such congenital malformations are diagnosed with CAH. Determining the appropriate sex in these patients requires urgent attention, particularly in CAH to evaluate life threatening situation such as salt wasting crisis [(1)]. Ambiguous genitalia in the newborn and children needs prompt attention for gender assignment which should be guided according to the

underlying etiology of the genital anomalies [(2)].Such normal sexual development abnormalities are relatively common and occurs in 1/4500 births approximately [(3)].

The role played by Y chromosome, when it comes to sex determination has been established since the 1950s [(4)]. It is the Y chromosome, presence or absence of which influences the sex determination process in an embryo [(5)]. XY embryos turn into males and XX embryos into females. But, certain individuals with a Y chromosome have been identified who phenotypically resemble a female (46, XY female) and some have been identified with a normal female karyotype but phenotypically resemble male (46, XX males). 46XX males were described initially by 3 investigators in 1964 [(6,7)]. 46, XX maleness constitutes of a rare sex reversal syndrome which is estimated to affect 1 in 20,000-25,000 newborn males [(8)]. SRY gene was discovered on molecular analysis of such sex reversed patients [(9)].

The current study's aim was to investigate 100 children with ambiguous genitalia to check 17OH progesterone level for CAH and to cytogenetically assess the pediatric cases which were followed by molecular analysis. This study evaluated 5-46, XX and 6-46, XY sex reversed patients for Y chromosomal loci (SRY) for better understanding of molecular mechanism for sex determination in such cases. PCR and sequencing based assay for one of the CAH case was done for CYP21A2 gene mutation analysis.

## MATERIALS & METHODS

In this study, 100 pediatric patients were analyzed who were referred to Department of Pediatrics, Pediatric Research and Genetics Laboratory (Maulana Azad Medical College) (2016-2023) with age ranging from 3 days to 11 years with mean age of 11months. The study included children who were born with ambiguous genitalia at either the newborn nursery, Department of Pediatrics, Lok Nayak Jai Prakash hospital, New Delhi, or were referred from various other hospitals for investigation from New Delhi. The approval by the institutional ethical committee and the informed consent was taken.

### BIOCHEMICAL ANALYSIS-

17-hydroxyprogesterone(17-OHP) level was checked to evaluate CAH (Congenital Adrenal Hyperplasia) by using Victor 2D-Fluormeter (PerkinElmer). 17-OHP level in serum in used as criteria for diagnosing CAH for 21-hyrdoxylase deficiency (21-OHD) [(10), (11)].

### CYTOGENETIC ANALYSIS-

0.5 ml of blood sample was collected and stored in heparinized vial followed by culturing of lymphocytes and incubation at 37-degree C for 72 hours. Colcemid was added to arrest cell division at metaphase. Harvesting was done by adding 7 ml of hypotonic KCl (0.562%) solution. The cells were fixed with the fixative 3:1 (Methanol: Acetic acid) after discarding the obtained supernatant. The slides were prepared and stained with a 4% giemsa stain. All subjects underwent chromosomal analyses wherein karyotyping was done with the help of Cytovision Version 3.9 software by analyzing 30 well-spread and well banded metaphases using GTG banding techniques (350 band resolution) [(12)]. Chromosomal analysis was done in accordance to International System for Human Cytogenetic Nomenclature (ISCN,2013) [(13,14)].

### MOLECULAR ANALYSIS-

2-3ml blood sample was collected in Ethylenediamine tetraacetic acid (EDTA) vials. DNA was extracted from the collected blood sample by phenol chloroform method. Primers were used to amplify target SRY region (on Y chromosome). Primers used-Forward-5'GAATATTCCCGCTCTCCGGAG3', Reverse-5'ACC TGTTGTCCA GTTGCACT3' [(15)]. Amplification was done by following the standard protocol-Initial Denaturation (94 degree C,7 minutes), Denaturation cycles, (94 degree C,30

seconds), annealing(55 degree C),extension(72 degree C). PCR products were run on agarose gel using ethidium bromide and Gel Docsystem was used for taking photographs. For CYP21A2 gene mutation analysis, PCR and sequencing based assay was carried out.

## RESULT-

During the last 7 years, 100 pediatric patients were referred to the Pediatric Research and Genetic laboratory for genetic diagnostics. Upon biochemical analysis,4 pediatric patients were found with elevated 17-OHP levels.

For CYP21A2 molecular analysis of 4 pediatric patients with elevated 17-OHP levels showed c. 1222+1G>T homozygous pathogenic variant in intron 9 of CYP21A2 in one patient, two patients showed homozygous CYP21A2;6:rs6467:C>G intron pathogenic variant and one patient showed Exon 3 deletion in CYP21A2.

Upon cytogenetic analysis,4 cases (4%) demonstrated mosaicism and the rest 96cases (96%) demonstrated 46, XX or 46, XY karyotype. 31cases (31%) had 46, XX karyotype and 65cases (65%) had 46, XY karyotype (TABLE 1).

**TABLE 1- CYTOGENETIC RESULTS**

| SNo | CYTOGENETIC RESULT | SOCIAL SEX | NUMBER OF CASES n (%) |
|---|---|---|---|
| 1. | 46, XX/46, XY | M | 2(2%) |
| 2. | 45, XO/46, XX | F | 1(1%) |
| 3. | 45, XO/46, XY | F | 1(1%) |
| 4. | 46, XX | M | 5(5%) |
| 5. | 46, XY | F | 6(6%) |
| 6. | 46, XX | F | 26(26%) |
| 7. | 46, XY | M | 59(59%) |

Some individuals carried a Y chromosome but phenotypically resembled a female and some depicted a female karyotype but were phenotypically male. Eleven sex reversed cases [5- 46, XX and 6- 46, XY] were successfully analyzed for SRY gene. Out of the 11 sex reversed cases 7 cases were detected positive for SRY gene (TABLE 2).

CYP21A2 Molecular analysis : 4 pediatric patients with elevated 17-OHP levels showed c. 1222+1G>T homozygous pathogenic variant in intron 9 of CYP21A2 in one patient, two patients showed homozygous CYP21A2;6:rs6467:C>G intron pathogenic variant and one patient showed Exon 3 deletion in CYP21A2.

**TABLE 2- Molecular screening for SRY**

| SNo. | AGE(Days) | SOCIAL SEX | SRY | CYTOGENETIC RESULT |
|---|---|---|---|---|
| 1 | 30 | F | +ve | 46, XY |
| 2 | 4 | F | +ve | 46, XY |
| 3 | 30 | F | +ve | 46, XY |
| 4 | 3 | F | +ve | 46, XY |
| 5 | 8 | M | -ve | 46, XX |
| 6 | 3 | M | +ve | 46, XX |
| 7 | 6 | M | -ve | 46, XX |
| 8 | 30 | M | -ve | 46, XX |
| 9 | 57 | F | +ve | 46, XY |
| 10 | 9 | F | +ve | 46, XY |
| 11 | 32 | M | -ve | 46, XX |

## DISCUSSION

The complexity revealed by the cytogenetic analysis reflects the complexity of the ambiguity. Normal sexual development comprises of various sequential events where an embryo progressively acquires male or female characteristics and formation of genital tract, external genitalia take place. Disturbance in any of these of these events can lead to disorders concerning sexual development. Although the etiology in such disorders is not clearly known, most appear

to be the result of sporadic errors in meiosis that produce chromosomal abnormality by sending a confused or aberrant signal to the process of gonadal differentiation. Such disorders associated with many types of sexual abnormalities can be a result of different genetic, endocrinological and environmental factors.

Mosaicism was reported in 4(4%) cases in our study. 45, XO/46, XY mosaicism is a rare DSD(Disorder of sexual development) and patients with this condition can be associated with broad range of phenotypes like females in Turner syndrome, males with gonadal dysgenesis , pseudohermaphroditism [16, 29]. Such patients are at an elevated risk of production of a cytogenetically abnormal offspring with chromosomal anomalies due to production of aneuploid sperm [17]. About 4-6.9% females with Turner syndrome exhibit Y chromosome mosaicism [18,19]. 46, XX/46, XY and 45,XO/46,XX mosaicism was also reported in our study which represented cells with different genotypes.

We also evaluated 11 sex reversed cases, out of which 7 were found positive with SRY gene. Human sexual differentiation being a highly complex process is guided by multiple genes, hormones. Molecular analysis has revealed that about 90% of 46,XX karyotype patients have varying Y material amount because of an illegitimate recombination which occurs during paternal meiosis resulting in Y to X exchange [20]. Kusz et al in 1999 demonstrated that a Y to X translocation, inactivation of Y bearing X chromosome in XX males could be responsible for ambiguous sexuality [21]. 3 mechanisms could be contributing towards the genesis of male phenotype in XX males-translocation in Y sequence, mutation in autosomal or X linked gene responsible for testis determining pathway, or Y chromosome mosaicism [22]. XX males can be Y+ve or Y- ve based upon the presence and absence of Y- derived specific sequence. Normal pubertal development is not seen in most XX males. We detected SRY gene's presence in one pediatric patient with 46, XX karyotype and male phenotype. This could have been a result of X to Y translocation. A similar case has been reported in other studies as well [15,23].

About 90% of the CAH patients have CYP21A2 gene mutations, large deletions or conversion to pseudogene (CYP21A1P) [24]. There is up to 95% and 98% sequence homology between CYP21A2 and CYP21A1P. This sequence homology along CYP21A2 locus's high variability results in crossing over events or CYP21A2 deletion [25,28]. CYP21A2 gene mutational analysis becomes challenging because of highly homologous psuedogene. Identifying CYP21A2 mutations is a critical step in early detection. In our study, upon conducting PCR and sequencing based assay in one of the CAH case, we observed Intron 2 Splice site C>G (Homozygous) mutation.

## LIMITATIONS AND STRENGTH

Although a number of diagnostic algorithms exist for DSD classification, no single evaluation protocol is suitable for all circumstances and some basic tests, such as hormone assay, ultrasonography and cytogenetic analysis are very important for classification and management of DSD. Further studies, using molecular genetic analyses particularly whole-genome sequencing are needed to give a more precise diagnosis. This study will strengthen the proper management of DSDs and will facilitate the sharing of experiences, thereby reducing the stress and isolation felt by patients and their families.

## CONCLUSION

Cytogenetic studies at the chromosomal level for the newborn with ambiguous genitalia need more investigation to support the results using PCR analysis (SRY gene) which provides information about the existence of Y chromosome. Molecular cytogenetics can provide useful insights and can assist in prognosis and better genetic counseling. The physician should help by providing appropriate genetic counseling to

enable the parents to make an early decision and to minimize the distress of these families. Despite all the odds a number of individuals with DSD are highly resilient, true to the words of Helen Keller 'Although the world is full of suffering, it is also full of overcoming it'

### *Declaration by Authors*

**Ethical Approval:** Approved

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