

## Molecular and Cytogenetic Evaluation of Gender in Patients Born with Ambiguous Genitalia from Different Regions of the Valley of Kashmir, North India

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### Abstract

Ambiguous Genitalia, a rare genetic disorder is caused by defects in the process of fetal sexual determination and differentiation where a newborn needs prompt evaluation to detect lethal conditions and gender assignment. This genetic screening is taken up with an aim to evaluate the prevalence of the patients born with Ambiguous Genitalia existing in our population.

We evaluated the patients for Ambiguous Genitalia with Polymerase Chain Reaction (PCR) using SRY gene and cytogenetic analysis for detecting X and Y chromosome was performed by conventional Karyotyping. Of the 50 Ambiguous Genitalia cases, 21 (42.0 %) were detected positive for SRY gene with 46, XY Karyotype and were recognized as males while as rest of cases 29 of 50 (58%) were confirmed as females by negative SRY gene coupled with 46, XX Karyotype. All the 21 cases positive for SRY gene were cytogenetically evaluated with 46, XY except two cases, one of testicular feminization syndrome and another case of AIS where a Karyotype revealed 46, XY but were negative for SRY gene. 09 of the 50 cases (18%) had undetermined sex and among these 05 (55.5%) were found to be negative for SRY gene with 46, XX Karyotype except one case with 47, XX which was an Edward's syndrome. 04 of the 09 cases (45.5%) were detected as males with positive SRY gene. Overall 04 cases (8.0 %) that were socially recognized as female for two decades were confirmed to be carrying Y chromosome as depicted by positive SRY gene with 46, XY Karyotype. We found that newborn with Ambiguous Genitalia exist in our region in a good number and the cytogenetic method coupled with molecular analysis by PCR are essential to unfold the sex and help in proper assignment of gender in intersex disorders.

**Keywords:** Ambiguous genitalia; SRY gene; Karyotype; Undetermined sex; Intersex disorders; Kashmir

### Introduction

Abnormalities in normal sexual differentiation are relatively common and occur in approximately 1 per 4500 live births [1]. Intersex disorders are rare congenital malformations and the reported incidences of the different malformations with over 80% being diagnosed with Congenital Adrenal Hyperplasia (CAH) in addition to other causes like True Hermaphroditism, Gonadal dysgenesis, Pseudohermaphroditism. Ambiguous Genitalia in the newborn and children need immediate and rational management and assignment of sex for rearing should be guided by the etiology of the genital malformation [2, 3].

The main factor influencing the sex determination of an embryo is the genetic sex determined by the presence or absence of the Y chromosome [4]. As a general rule, XY embryos become males and XX embryos become females. However, some individuals carry a Y chromosome but are phenotypically female (46, XY females) or have a female Karyotype but are phenotypically male (46, XX males). 46, XX maleness is a rare sex reversal syndrome affecting 1 in 20,000 newborn males. Abnormalities in normal sexual differentiation are relatively common and occur in approximately 1 per 4500 live births.

Human males with a 46, XX Karyotype were first described in 1964 by three different groups of investigators [5,6]. The frequency of this syndrome has been estimated to be 1 in 20,000-25,000 newborn males, although there are considerable geographic variations [7]. Molecular analyses have demonstrated that approximately 90% of patients with 46, XX karyotype carry a variable amount of Y material due to a Y-to- X interchange originated by an illegitimate recombination

during paternal meiosis [8]. In 1999, Kusz et al. demonstrated that in XX males with Y-to-X translocations, preferential inactivation of the Y-bearing X chromosome could be the major mechanism causing a sexually ambiguous phenotype [9]. The origin of male phenotype in XX males could be the result translocation of Y sequences, including the SRY gene, to an X chromosome or to an autosome; a mutation in a yet unknown X-linked or autosomal gene in the testis-determining pathway, and cryptic Y chromosome mosaicism [10]. External genitalia are underdeveloped and the presence of small testes is a distinctive trait of the syndrome [7]. In approximately 15% of XX males Hypospadias, Cryptorchidism, or severe genital ambiguity is observed [11].

The genetic sex of the embryo is established at fertilization, the phenotypic sex determining process is set in motion during the period of organogenesis when the gonads develop. Apart from sex specific genes present on X and Y chromosomes and some autosomal genes also play a role in sex determination [12]. Any alteration in the genes, gene dosage or the sex chromosomes lead to abnormalities of sexual

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development, ranging from complete sex reversal to Hermaphroditism. Sex determination and development in humans is a complex process, involving a cascade of molecular events. SRY is the gene coding for TDF and is therefore, essentially, the gene imparting maleness. It can be regarded as a genetic switch controlling male development.

This study was conducted with an aim to determine the prevalence of the newborn children and young patients with Ambiguous Genitalia existing in Kashmir (North India). We evaluated the patients for Ambiguous Genitalia with Polymerase Chain Reaction (PCR) using SRY gene and cytogenetic analysis for detecting X and Y chromosome was performed by conventional Karyotyping.

## Method

### Sample collection

The subjects included 50 patients with Ambiguous Genitalia referred for chromosomal and SRY gene analysis to Advanced Centre for Human Genetics, Sher-I-Kashmir Institute of Medical Science (SKIMS), Srinagar and the screening was conducted between 2012 and 2015. The cases were referred from Department of Endocrinology, Pediatrics and various OPD clinics of the hospital. Detailed pedigree analysis and in depth clinical evaluation and clinical reports were obtained from all subjects. The patients were accepted on the basis of physical features, hormone profile and radiological investigations. 2 to 3 ml peripheral blood in EDTA vials was collected for molecular analysis using PCR while as 1 to 2ml in heparinised syringes for cytogenetic analysis

#### DNA extraction and polymerase chain

Genomic DNA rpm peripheral blood was isolated using standard *Proteinase-K* digestion, phenol/chloroform extraction, and ethanol precipitation method from whole-blood samples.

### Molecular analysis (Polymerase chain reaction)

PCR was carried out in a final volume of 25  $\mu$ L containing 50 ng genomic DNA template, 1X PCR buffer (Biotools, B & M Labs, Madrid, Spain) with 2 mmol/L MgCl<sub>2</sub>, 0.4 mmol/L of each primer (Genscript, Piscataway, NJ), 50 mmol/L dNTPs (Biotools, B & M Labs), and 1 U *Taq polymerase* (Biotools, B & MLabs). Primers used for the amplification of the target SRY region of Y-chromosome were F: 5'-GAATATTCCTCCGCTCTCCGGAG-3' R: 5'-ACC TG TTGTCCA GTTGCAC-3'. For PCR amplification, the standard protocol was used as follows: one initial denaturation step at 94°C for 7 minutes, followed by 35 denaturation cycles of 30 seconds at 94°C, 30 seconds of annealing at 55°C, and 30 seconds of extension at 72°C, followed by a final elongation cycle at 72°C for 5 minutes. For quality control, each PCR reaction used male positive control DNA and a female negative control DNA for every run. The PCR products were separated by electrophoresis on 2% agarose gel containing ethidium bromide and photographed using Gel Doc system (Flourchem HD2, Cell Bioscience). A product size of 419 bp reveals a positive band for presence of Y chromosome.

### Cytogenetic analysis (Conventional Karyotyping)

Peripheral blood cultures were immediately initiated. Blood culture and slide preparation were performed according to Jyothy et al. [13]. 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) (Gibco, Invitrogen) in sterile tubes. The tubes were cultured at 37°C for 72 h. 100  $\mu$ L of colchicine (0.45 mg/ml) was added to each culture. After 60 min, the cells from

all culture tubes were harvested by centrifugation (2,000 rpm/10 mins). The supernatants were discarded and the cells re-suspended with the remaining solution. The cells were exposed to mild hypotonic treatment (3 ml of 0.075 M KCL). The cells were concentrated by another centrifugation and the pallet was re-suspended with remaining hypotonic solution and fixed with 5 ml fixative solution (3 Methanol: 1 Glacial acetic acid). The cell suspension was washed four times by repeated centrifugation and fixation at intervals of 20 min and finally slides were prepared. Then, the slides were treated with trypsin for 20–30 s, stained for 10 min 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. Karyotyping was performed with the help of Cytovision Version 3.9 software

## Results

The study included 50 patients referred to the Advanced Centre for Human Genetics, Sher-I-Kashmir Institute of Medical Science (SKIMS), for both molecular and chromosomal analysis to rule out Ambiguous Genitalia. The conventional Karyotyping was used as method for screening the patients with chromosomal status while as polymerase chain reaction (PCR) was used as a molecular tool to detect the presence of SRY gene. All the cases screened between 2012 and 2015 belonged to the different regions of Kashmir (J & K State, India).

Among 50 cases of Ambiguous Genitalia 27 (54.0 %) cases were of 0 to 3 years of age while as 09 (18.0 %) belong to 4 to 7 years of age. 35 (70.0 %) cases belonged to rural as against 15(30.0 %) from urban population. 10 (20%) patients were products of consanguineous marriage while as 40 (80.0 %) belonged to non- consanguineous marriage (Table 1). Among prominent clinical characteristics observed

Variable	Cases (n=50)	Percentage (%)
<b>Age group</b>		
0-3 Years	27	54.0
4-7 Years	09	18.0
8-11 Years	02	04.0
12-15 Year	02	04.0
≥16	10	20.0
<b>Dwelling</b>		
Rural	35	70.0
Urban	15	30.0
<b>Consanguinity</b>		
Yes	10	20.0
No	40	80.0
<b>Clinical condition</b>		
Hypospadias	08	16.0
AIS	04	08.0
CAH	07	14.0
Inguinal swelling	06	12.0
Clitoromegaly	07	14.0
Turner's syndrome	01	02.0
Edward's syndrome	01	02.0
Primary amenorrhea	04	08.0
Male pseudohermaphroditism	02	04.0
Testicular feminization syndrome	01	02.0
Others	09	18.0

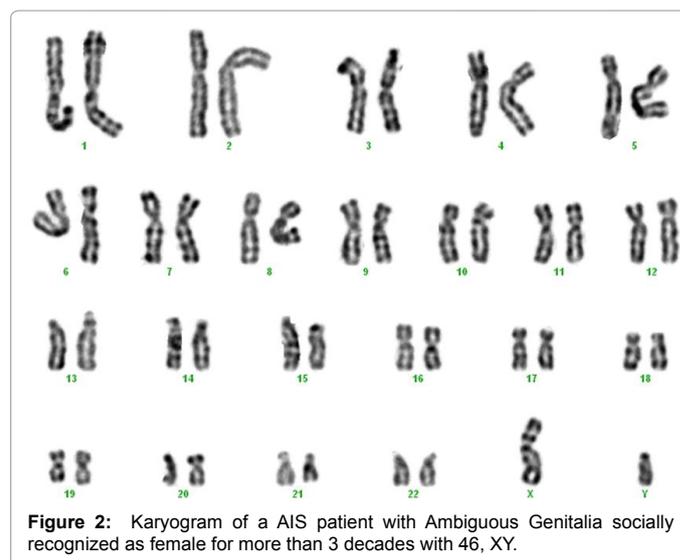
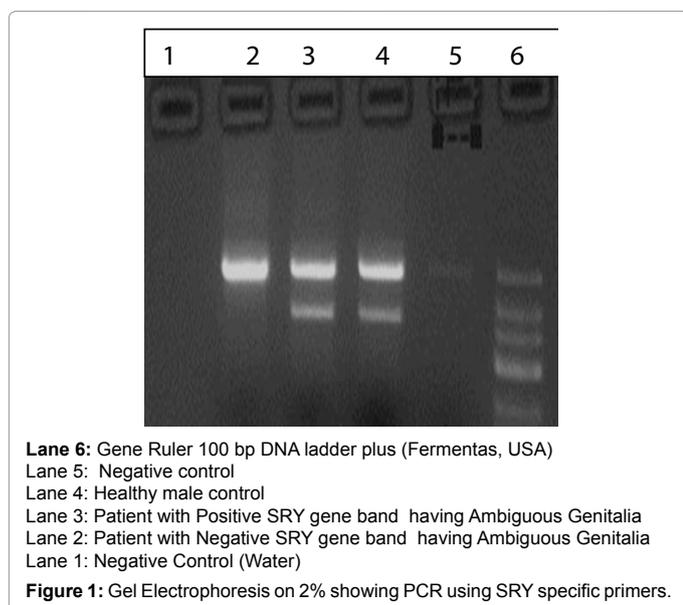
CAH: Congenital Adrenal Hyperplasia

**Table 1:** Demographic and clinical characteristics of Ambiguous Genitalia patients

in cases with Ambiguous Genitalia, Hypospadias was found in 08 cases (16.0%), Androgen Insensitivity Syndrome (AIS) 04(8.0%), Congenital Adrenal Hyperplasia (CAH) 07 (14.0%), Inguinal Hernia 06 (12.0%) and Clitoromegaly 07 (14.0%) (Table 1).

Of the 50 Ambiguous Genitalia cases 21 (42.0 %) were detected positive for SRY gene and were recognized as males while as rest of cases 29 of 50 (58%) were confirmed as negative for the SRY gene, thus recognized as females (Table 2). All the 21 cases positive for SRY gene were cytogenetically evaluated with 46, XY except one case, that of testicular feminization syndrome where a Karyotype revealed 46, XY but was negative for SRY gene. All the other 29 cases with SRY negative status were depicted as 46, XX Karyotype except one case of AIS where SRY gene was found positive. 09 of the 50 cases (18%) entertained from age group 16 days to one year were not assigned any gender and therefore, had undetermined sex. Among these 05 of 09 (55.5%) cases were found to be negative for SRY gene by PCR and cytogenetics revealed 46, XX Karyotype except one case with 47, XX which was an

Edward's syndrome. 04 of the 09 cases (45.5%) with undetermined sex were detected as males with positive SRY gene and all had cytogenetic profile as 46, XY (Table 2). Among 05 cases of CAH with undetermined sex, 02 were found to be SRY positive with 46, XY Karyotype and 03 cases with SRY negative having 46, XX Karyotype. In 03 cases of AIS with female phenotype, two cases showed diagnostic conformity by Karyotyping and PCR with one detected as 46, XY and SRY positive status while as the other vice versa. The third case of AIS interestingly was found with 46, XY Karyotype but SRY gene was absent (Figure 1). Further 03 cases of primary amenorrhea that were more than 20 years age and socially recognized as females with ambiguity in their genitals revealed opposite gender with 46, XY Karyotype having positive SRY gene. Overall 04 cases (08.0 %) that were socially recognized as female for two decades were confirmed to be carrying Y chromosome as depicted by positive SRY gene with all having 46, XY Karyotype (Figure 1) (Figure 2) (Table 2) and on their radiological examination revealed absence of their ovaries and uterus.



S.No	Case #	Clinical Condition	Age	External Genitalia	Social Sex	SRY	Karyotype
01	SRY-02	Mullerian dysgenesis	1 M	Ambiguous	UD	-ve	46, XX
02	SRY-13	Testicular feminization syndrome	11 Y	Ambiguous	F	-ve	46,XY
03	SRY-14	Primary amenorrhea	37 Y	Ambiguous	F	+ve	46, XY
04	SRY-12	Primary amenorrhea	20 Y	Ambiguous	F	+ve	46, XY
05	SRY-18	AIS	15 Y	Ambiguous	F	+ve	46, XX
06	SRY-19	Clitoromegaly	8 Y	Ambiguous	F	+ve	46, XY
07	SRY-24	AIS	16 Y	Ambiguous	F	-ve	46, XX
08	SRY-31	AIS	36 Y	Ambiguous	F	+ve	46, XY
09	SRY-36	Male Pseudohermaphroditism	16 Y	Ambiguous	F	+ve	46, XY
10	SRY-46	Edward's syndrome	1M	Ambiguous	UD	-ve	47, XX
11	SRY-21	CAH	1M	Ambiguous	UD	-ve	46, XX
12	SRY-34	CAH	22 D	Ambiguous	UD	+ve	46, XY
13	SRY-16	CAH	16 D	Ambiguous	UD	-ve	46, XX
14	SRY-39	CAH	1M	Ambiguous	UD	+ve	46, XY
15	SRY-05	Aphallic	3M	Ambiguous	UD	+ve	46, XY
16	SRY-44	CAH	15 D	Ambiguous	UD	-ve	46, XX
17	SRY-31	Cryptochidism	1 Y	Ambiguous	UD	+ve	46, XY

F: Female, UD: Undetermined, CAH: Congenital Adrenal Hyperplasia, AIS: Androgen Insensitivity Syndrome, M: Months, Y: Years, D: Days

**Table 2:** Distribution of Clinical, Molecular and Cytogenetic findings of ambiguous patient groups.

## Discussion

Among other genetic disorders prevalent in this region Ambiguous Genitalia is commonly found here and a number of cases harboring the same condition have been evaluated. For appropriate and effective management and counseling of patients, cytogenetic confirmation and knowledge of the development of the genital tract and of the interaction between genetic sex and environment is mandatory [4]. The main factor influencing the sex determination of an embryo is the genetic sex determined by the presence or absence of the Y chromosome [14]. As a general rule, XY embryos become males and XX embryos become females. However, some individuals carry a Y chromosome but are phenotypically female (46, XY females) or have a female Karyotype but are phenotypically male (46, XX males). Ambiguous Genitalia is a social & medical emergency and it needs prompt evaluation to detect life-threatening conditions such as salt-losing crisis in CAH and gender assignment. The ambiguity in Differentiation Sex Disorder is characterized both by molecular technique (PCR) as well as cytogenetic analysis (Karyotyping). Karyotyping is systematic while PCR analysis of the SRY gene provides information about the presence of a Y chromosome within 1 day [15]. However, PCR based sex determination is rapid, reliable and economic and provides an accurate means of determining sex of an individual, including the detection of hidden Y sequence [16,17]. We evaluated the patients for Ambiguous Genitalia with PCR using SRY gene and cytogenetic analysis for X and Y chromosome was performed by conventional Karyotyping.

We detected 21 cases as positive for SRY gene that were cytogenetically confirmed with 46, XY Karyotype. Among them two cases were negative for SRY gene, one of testicular feminization syndrome and another case of AIS where both revealed 46, XY Karyotype. All the other 29 cases with SRY negative status were depicted as 46, XX Karyotype.

Kaur and Singh, 2010 conducted cytogenetic investigations of 1950 cases with genetic disorders, 9.7% cases were presented with sex anomalies [18]. Ambiguous Genitalia and Hypogonadism constituted 4% cases and the total frequency of sex anomalies was 9.7%. Earlier Kaur et al. 2004 investigated 156 cases with varied abnormalities of sexual development that showed a total of 40 cases (25.6%) abnormal Karyotype [19].

Rajendran and Hariharan studied thirty five children with Ambiguous Genitalia where 18 cases were assigned female sex. In our study, 18% (09/50) cases from age group 16 days to one year, had undetermined sex [20]. Among these 05 (55.5%) cases were found to be negative for SRY gene by PCR with 46, XX Karyotype except one case with 47, XX which was an Edward's syndrome. 04 cases (45.5%) with undetermined sex were detected as males with positive SRY gene bearing cytogenetic profile as 46, XY.

Although majority of 46, XX true hermaphrodites were negative for the Y-DNA sequence, including SRY sequence a minority of individuals were found to be positive for SRY sequence [21-23]. However, there was no report of Y specific SRY sequence in female Pseudohermaphrodites. In this report we found one male Pseudohermaphrodite who had been initially been raised as female and was detected with 46 XY Karyotype having positive SRY gene.

In 03 cases of AIS with female phenotype, two cases showed diagnostic conformity by Karyotyping and PCR while the third case of AIS interestingly was found with 46, XX Karyotype but SRY gene was present. Molecular analyses have demonstrated that approximately 90%

of patients with 46, XX Karyotype carry a variable amount of Y material due to a Y-to-X interchange originated by an illegitimate recombination during paternal meiosis [24]. In 1999, Kusz et al. demonstrated that in XX males with Y-to-X translocations, preferential inactivation of the Y-bearing X chromosome could be the major mechanism causing a sexually ambiguous phenotype [9]. Similar study by Huseyin Y et al in 2008 detected complete AIS syndrome phenotype in 14.3% patients with 46, XY Karyotype but had not conformed these to SRY gene analysis by PCR [25].

In this report yet another clinical presentation for Ambiguous Genitalia was Congenital Adrenal Hyperplasia where among 07 patients, 05 had undetermined social sex. In this group, we found 03 patents as females confirmed by positive SRY gene and 46, XX Karyotype and 02 as vice versa. Few studies have shown cases with CAH resulting from 21-hydroxylase deficiency identified with negative SRY by PCR with over masculinized genitalia in females having 46, XX Karyotype [26-28].

Gender identity is a complex process of differentiation that is affected by numerous variables. The gender assignment of intersex patients is related to social, cultural and religious factors to and timely intervention is essential for proper genetic counseling and their normal development. Finally our report gives evidence that PCR based DNA diagnostic method using SRY gene in combination with conventional cytogenetics play an important role for sex determination of individuals with Ambiguous Genitalia and to acquire more knowledge on the molecular mechanism of sex determination

## Conclusion

In conclusion the cytogenetic method coupled with molecular analysis by PCR of children with Ambiguous Genitalia is essential to unfold the genotype and help in proper assignment of gender in intersex disorders. We observed the two techniques complement each other to overcome the discordance and thus help in detecting the nature of the reason in deducing the ambiguity of sex.

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