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Anatomy Section

A Study of Cytogenetic Pattern in Amenorrhea

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ABSTRACT

Introduction: Menstruation is an important physiological function of female reproductive system during reproductive age. Prevalence of amenorrhea is higher in Indian population with a potential social impact. The study of cytogenetic pattern in its correlation with other physiological factors will help for better understanding the cause and earlier diagnosis.

Aim: To find out cytogenetic pattern among amenorrhea patients and to correlate with the phenotype and clinical findings among these cases.

Materials and Methods: This study was performed on 200 women volunteers after taking an informed consent. Out of 200 cases 100 cases were grouped as controls and 100 cases as study group. Cytogenetic study was done by conventional karyotyping and other clinical investigations (hormonal assay, fasting blood glucose) were also carried out.

Results: In the present study the frequency of classic Turner's (25%) and mosaic Turner's (12.5%) among primary amenorrhea cases. All Secondary amenorrhea cases were with normal chromosomal complement that is 0% frequency of chromosomal aberrations. One case with polycystic ovarian disease exhibited Premature Chromatid Separation (PCS).

Conclusion: Identification of known genetic causes could aid in development of effective treatments for women with amenorrhea, as well as earlier diagnosis which may allow for family planning before the onset of amenorrhea.

Keywords: Imperforate hymen, Mullerian dysgenesis, Premature chromatid separation, Turner's syndrome, X-chromosome

INTRODUCTION

In our country a married woman's social standing is determined by her ability to bear a child. Barrenness or inability to bear a child is considered a social stigma. To have an offspring, a woman should have physiologically well functioning reproductive organs. Menstruation is an important physiological function of female reproductive system. According to American Society for Reproductive Medicine the terms primary and secondary amenorrhea describe the occurrence of amenorrhea after menarche as secondary amenorrhea and not attaining menarche as primary amenorrhea [1].

Amenorrhea is considered as a symptom and not a disease and is a feature in conditions like gonadal anomalies, endocrinological and genetic disorders [2].

The incidence of menstrual irregularity is 5%, indicating that menstrual disturbance is a persisting problem among Indian women [3]. With a potential social impact and higher prevalence of amenorrhea in Indian society; extensive evaluation is required to overcome the anxiety.

Integration of hormones from hypothalamus, pituitary and ovary is required for regular menstrual cycles. Normal menstruation requires anatomically normal reproductive tract and a genetically normal chromosomal compliment of 46, XX [4]. The present study is performed to find out cytogenetic pattern among amenorrhea patients and to correlate with the phenotype among these cases.

MATERIALS AND METHODS

This case-control study was performed on 200 women volunteers after taking an informed consent for a period of three years, 2013 to 2016. The volunteers were outpatients attending the OPD of Vinayaka Missions Medical College Hospital Salem. Tamil Nadu, India. Ethical clearance and approval for the study was obtained from Institutional Ethics Committee and Institutional Review Board of Vinayaka Missions Medical College. Out of 200 cases 100 cases, were grouped as controls and 100 cases as study group according to following criteria-

Inclusion criteria for study group: (ASRM, 2008) [1]

- Age: 16 to 40 years old females.
- Patients presenting with primary amenorrhea due to non-attainment of menarche.
- Patients presenting with secondary amenorrhea due to following causes: anatomic defects of the genital tract, hypothalamic/pituitary causes, endocrinopathies, chronic oligomenorrhea or anovulation, polycystic ovarian syndrome, premature ovarian failure.

Inclusion criteria for control group:

- Age: 16 to 40 years old females.
- Women with the history of regular menstrual cycles.
- Women with normal serum hormonal levels.

Exclusion criteria for both study and control groups:

- Age: Women below 16 years and above 40 years.
- Pregnant and lactating mothers.
- Women undergoing any treatment with medication or drugs effecting menstrual cycle.
- Women having history of surgical treatment in relation to genital tract.

Diagnosing primary and secondary amenorrhea was carried out with the help of detailed history, physical examination and laboratory testing [4].

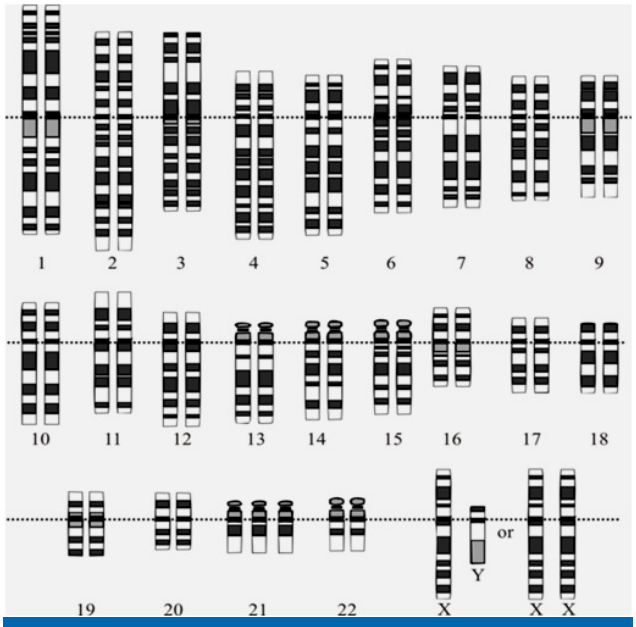
Cytogenetic analysis: Work flow of karyotyping according to The AGT Cytogenetics Laboratory Manual [5]:

Sample collection → Culture → Harvesting → Slide preparation → Staining → Interpretation.

Peripheral blood was collected in a sterile heparin coated vacutainers with aseptic precautions. Blood sample was inoculated in culture tube containing RPMI 1640 culture medium, Phytohaemagglutinin (PHA) and autologous plasma. The cultures were incubated in 37^{0}C for 72 hours. The cell division was arrested in metaphase stage of cell cycle by using Colchicine solution. Hypotonic solution treatment was done by using potassium chloride solution. The cells were fixed by using fixative, after three to four washes with fixative, the slides were prepared by dropping two to three drops of cell suspension from two feet height over a cleaned slide. The staining was done by conventional GTG banding. The stained slide was analysed based on [Table/Fig-1]. Chromosomes were classified and interpreted.

RESULTS

Based on the diagnosis the study group was categorised into four sub-groups: Individuals with- 1) Primary amenorrhea (PA) (n=8); 2) Secondary amenorrhea with unknown etiology (SA) (n=6); 3) Polycystic ovarian disease (PCOD) (n=82), Premature ovarian failure (POF) (n=4).



[Table/Fig-1]: Normal banding patterns of metaphase.

Cytogenetic analysis: Out of eight primary amenorrhea samples three samples were found to have an abnormal karyotype. Out of these three samples, two were classic Turner's syndrome having chromosomal complement 45, XO and one sample exhibited mosaicism 45, XO / 46, XXq-. In the present study normal chromosomal complement was found in all secondary amenorrhea (PCOD, POF, and secondary amenorrhea with unknown etiology) cases. But one case with PCOD exhibited PCS. Karyotype wise distribution of subjects among the cases and controls was demonstrated in [Table/Fig-2].

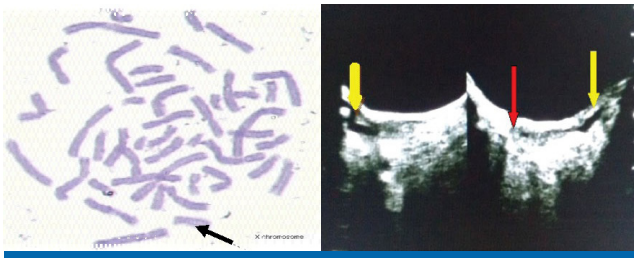
Case reports of Turner's syndrome cases

Case-1: Chromosomal complement: 45, XO [Table/Fig-3].

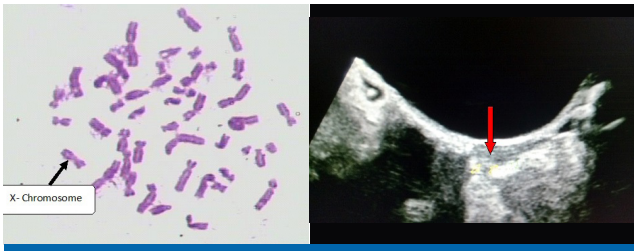
- Age: 17 years, Gender: Female
- BMI-17.5 Kg/m^2 (underweight)
- Hormonal levels: FSH-97.5 mIU/mL, LH-10.5 mIU/mL, PRL-13.5 ng/mL, TSH-10.5 µg/L, FSH: LH-9.28
- Phenotype was short stature, broad chest, lymphoedema of the hands and feet.

Karyotype	Cases	Controls	p-value (X2 by Yate's correction)
46 XX	97	100	<0.0001(HS)
46 XO	2	0	
45 XO/46XXq-	1	0	
Total	100	100	

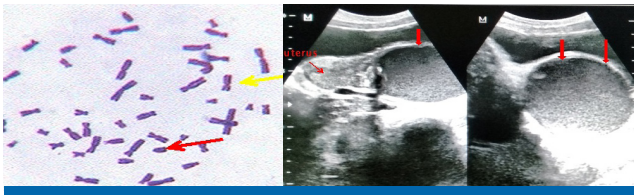
[Table/Fig-2]: Karyotype wise distribution of subjects among the cases and controls.
(*HS- highly significant)



[Table/Fig-3]: Metaphase spread of a Turner's syndrome patient with chromosomal compliment (45 XO) (left). **[Table/Fig-4]:** Streaky gonads (yellow arrow) and hypoplastic uterus (red arrow) (right).



[Table/Fig-5]: Metaphase spread of a Turner's syndrome patient with chromosomal complement (45 XO) (left). **[Table/Fig-6]:** Rudimentary uterus (red arrow) (right).



[Table/Fig-7]: Normal X- chromosome (yellow arrow); X chromosome with q arm deletion (red arrow) (left). **[Table/Fig-8]:** Imperforate hymen showing fluid filled vagina (red arrow) (right)

- Poorly developed secondary sexual characteristics. Mammary gland exhibits Tanner Stage-1.
- Ultrasonography report: Streak gonads, uterus rudimentary [Table/Fig-4] coarctation of aorta.

Case-2: Chromosomal complement: 45, XO [Table/Fig-5]

- Age: 26 years, Gender: Female
- BMI:18.5 Kg/m^2 (normal weight)
- Hormonal levels: FSH-100.8 mIU/ml, LH-5.9 mIU/ml, PRL-11.3 ng/ml, TSH-4 µg/L, FSH: LH-17.08.
- Phenotype shows short stature, poorly developed secondary sexual characteristics. Mammary gland exhibits Tanner Stage-2.
- Ultrasonography report shows rudimentary uterus measuring 1.6 x 0.5 cm. Ovaries not visualized [Table/Fig-6].

Case-3: Chromosomal complement: 45,XO/46,XXq- Table/Fig-7].

- Age: 20 years, Gender: Female
- BMI:18 Kg/m^2 (underweight)
- Hormonal levels: FSH-88.5 mIU/mL, LH-44.5 mIU/mL, PRL-14.5 ng/mL, TSH-2 µg/L, FSH: LH-1.98. Serum FSH and LH levels were very high than normal range. Serum PRL, TSH was within normal range and FSH: LH was less than 2.
- Phenotype showed short stature, poorly developed secondary sexual characteristics. Mammary gland exhibits Tanner Stage-3. Absence of axillary hair. External genital morphology was normal.
- Congenitally dumb and deaf.
- Ultrasonography report shows rudimentary uterus measuring 1.7 x 0.8 cm. Thin myometrium, minimal endometrial layer, presence of small cyst. Ovaries: Right ovary not visualised, left ovary was small measuring 1.3 x 0.5 cm.

Primary amenorrhea with normal chromosomal complement: Out of five cases of primary amenorrhea with normal chromosomal complement, three cases were found to have Mullerian agenesis and in one of these three cases congenital anomalies (polycystic right kidney, bilateral inguinal hernia, presence of bilateral cervical rib) were also associated with rudimentary uterus. Among rest of the two cases one case was reported as ovarian dysgenesis and another case as vaginal block (imperforate hymen) [Table/Fig-8]. Clinical features of patients with primary amenorrhea in present study was depicted in [Table/Fig-9].

A case of premature chromatid separation: In present study one secondary amenorrhea case with PCOD case was found to have premature chromatid separation (PCS) [Table/Fig-10], around 20% of metaphase spreads were exhibiting PCS with normal chromosomal complement (46,XX). Phenotypically normal built with normal secondary sexual characteristics.

DISCUSSION

Cytogenetic investigations are considered as most valuable and fundamental investigation in the diagnosis of amenorrhea. Frequency of sex chromosomal anomalies among amenorrhea cases in different studies is shown in [Table/Fig-11] [6-15].

In the present study the frequency of classic Turner's (25%) and mosaic Turner's (12.5%) among PA cases was similar when compared with previous studies which was ranging from 7%-46% . But, the frequency of chromosomal aberrations in SA cases was varying widely in different studies. In the present study all secondary amenorrhea (PCOD, POF, and secondary amenorrhea with unknown etiology) cases were with normal chromosomal complement that is 0% frequency of chromosomal aberrations.

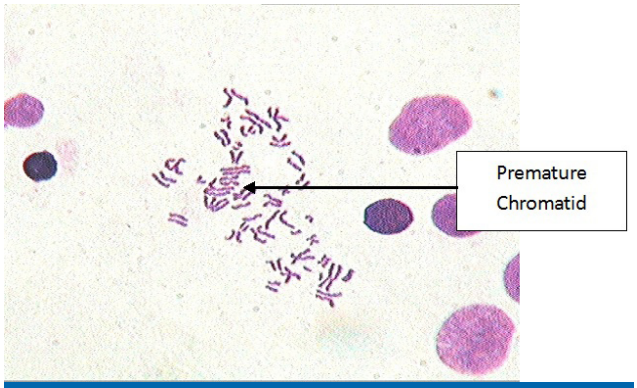
Frequency of classic and mosaic form of Turner's syndrome

among primary amenorrhea cases in various countries was shown in [Table/Fig-12] [11,14-23]. The figures quoted in this table shows a wide range of frequency distribution in different countries, the range of classic Turner's was 2%-63%, the range of the mosaic Turner's was 15%-66%. In the present study the percentage of classic Turner's was 25% and mosaic Turner's was 12.5%.

Comparison of frequency of sex chromosomal anomalies in present study with that of other studies available in the literature is depicted in [Table/Fig-13] [8,10,12,15]. The frequency of sex chromosomal aberrations reported by various researchers shows the percentage of PA ranging from 6% to 46% and the percentage of X- chromosomal aberrations in SA ranging from 0.4% to 16%. In the present study frequency

Clinical Features	Classic TS (45,XO)	Mosaic TS (45,XO/46, XXq-)	PA with Normal Karyotype (46, XX)	Total
No. of Cases	2	1	5	8
Short stature (<150 cm)	2	1	0	3
Webbed neck	0	0	0	0
Short neck	1	1	0	2
Breast developmental delay	2	1	0	3
Presence of rudimentary uterus/ absence of uterus	2	1	4	7
Absence of ovaries/ streak gonads	2	1	2	5
Raised FSH levels (>24 ng/mL)	2	1	4	7
Raised LH levels (>16 µg/L)	0	1	0	1

[Table/Fig-9]: Clinical features of patients with primary amenorrhea in present study.
*TS-Turner's Syndrome, †PA- Primary Amenorrhea, ‡ FSH-Follicle Stimulating Hormone, §LH-Luteinizing Hormone.



[Table/Fig-10]: Metaphase spread displaying premature chromatid separation.

of sex chromosomal aberrations among primary amenorrhea cases was 37.5% which was similar to the range of previous studies. The frequency of sex chromosomal anomalies among secondary amenorrhea cases was 0% which means that sex chromosomal anomalies among SA cases is very rare.

The phenotype presentation of the present case varied widely when compared with previously reported cases on Xq deletion [24].

Prevalence of Xq deletion has been found to be a rare structural aberration among PA cases shown in [Table/Fig-14] [11,24]. Mosaicism with Xq deletion was most uncommon sex chromosomal aberration which was seen in one PA case of the present study.

Author	Year	Total No. (n)	No. of PA (n)	No. of SA (n)	No. of PA with CA (%)	No. of SA with CA (%)
Goldman B et al., [6]	1982	107	63	44	10 (6.3%)	1(0.4%)
Opitz JM et al., [7]	1983	103	88	15	25 (28%)	5(33%)
Ten SA et al., [8]	1990	117	117	-----	36 (31%)	------
Goud IK et al., [9]	2006	58	58	-----	8 (14%)	------
Rajangam S et al., [10]	2007	865	620	245	161 (26%)	39(16%)
Zhao X et al., [11]	2008	131	131	------	48 (36.6%)	------
Kalavathi V et al., [12]	2010	979	852	127	221 (26%)	9(7%)
Laxmi KV et al,. [13]	2010	140	140	-----	39 (27.8%)	------
Jouyan N et al., [14]	2012	354	354	------	163(46%)	------
Datta UR et al., [15]	2013	637	251	28	132 (20.7%)	------
Present study	2016	100	8	92	3(37.5%)	0(0%)

[Table/Fig-11]: Frequency of sex chromosomal anomalies among amenorrhea cases in different studies.
*PA- Primary Amenorrhea, †SA-Secondary Amenorrhea, ‡CA-Chromosomal Aberrations.

Comparison of clinical features and karyotype distribution among PA cases is depicted in [Table/Fig-9]. In the present study the webbed neck condition was absent among PA cases. Distribution of other clinical features was found to be similar with the previous studies.

Premature chromatid separation: (MIM-176430)

Premature Chromatid Separation (PCS) refers to an autosomal dominant trait with separate chromatids and discernible split centromere effecting all chromosomes and is coupled with

heterozygous mutation in a gene BUB1B (MIM- 602860). This gene is responsible for the formation of mitotic spindles results in precocious separation of centromere occurs during metaphase causing cell division errors.

Country	Classic Turner (%)	Mosaic Turner (%)	Author
China	7	18.3	Zhao X et al., [11] 2008
Iran	34	66	Jouyan N et al., [14] 2012
India	19	17	Datta UR et al., [15] 2013
Brazil	29	53	Duarte AC et al., [16] 2004
Korea	2.1	50.8	Kim SS et al., [17] 1999
Italy	50	37	Nucaro AL et al., [18] 2008
Denmark	45	15	Nielsen J et al., [19] 1991
Minnesota	42	48	Wiktor AE et al., [20] 2005
Tunisia	32	47	Kammoun I et al., [21] 2008
Kuwait	63	22	Abulhasan SJ et al., [22] 1999
Singapore	57	---	Tan KB et al., [23] 2009
This study	25	12.5	---------

[Table/Fig-12]: Frequency of classic and mosaic form of Turner syndrome among primary amenorrhea cases in various countries.

Premature chromatid separation is often confused with a similar term called premature centromeric division which is characterised with a rod shaped X-chromosome and without a distinct centromere and this is due ageing among women [25].

Errors of cell division lead to formation of gametes which have more chances of non disjunction which could be a cause for spontaneous abortion, recurrent abortions and Down's syndrome child [26].

In present study one secondary amenorrhea with PCOD case was found to have premature chromatid separation.

Author	Year	Total No. of Cases	No. of Cases with 46, XXq- (%)	No. of Cases with 45,XO/46, XXq- (%)
Ten SA et al., [8]	1990	117	1(0.8%)	0(0%)
Rajangam S et al., [10]	2007	865	4(0.4%)	0(0%)
Kalavathi V et al., [12]	2010	979	1(0.1%)	0(%)
Datta UR et al., [15]	2013	637	3(0.4%)	2(0.3%)
Present Study	2016	100	0(0%)	1(1%)

[Table/Fig-13]: Comparison of mosaicism with Xq deletion and Xq deletion among amenorrhea patients.

The frequency of premature chromatid separation was 10% - 45% was observed among metaphase spreads in couples with a history of recurrent abortions [27]. In present study around 20% of metaphase spreads were exhibiting PCS. All cases with premature chromatid separation were having

Clinical Feature and Karyotype	Elsheikh, et al.,[25] 2002 (%)	Zhao et al.,[11] 2008 (%)	Present Study (%)
Short stature (<150 cm)	98	91	100
Webbed neck	25	3	0
Short neck	81	79	66
Breast developmental delay	-----	100	100
Presence of rudimentary uterus/ absence of uterus	-----	100	100
45,XO	48	27	66
Mosaicism	18	48	33

[Table/Fig-14]: Clinical features and karyotype distribution of TS patients compared with the data of other studies.

normal chromosomal complement 46, XX in females and 46, XY in males [28]. In present study a case of woman of 25 years of age, having PCS with a normal chromosomal complement was found. Hormonal levels (FSH, LH, Prl & TSH) were within normal limits and exhibited a normal phenotype. The Fasting blood glucose level was also normal. Metaphase spread of premature chromatid separation was shown in [Table/Fig-8].

LIMITATION

The limitation of present study was lack of karyotyping software analyser which helps to pair the chromosomes. Though, the bands of chromosomes were clear under microscope, but the bands were not well appreciated in the photographs taken. The availability of primary amenorrhea cases in high number for further extensive research curtails the study to an extent. Availability of more number of primary amenorrhea samples for the study would have validated the study from a different point of view.

CONCLUSION

Identification of known genetic causes could aid in development of effective treatments for women with amenorrhea, as well as earlier diagnosis which may allow for family planning before the onset of amenorrhea. Eliciting a proper history along with a meticulous clinical examination and investigations for chromosomal aberrations will provide a solid foundation for treatment of women with amenorrhea leading a fruitful reproductive life.

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