

Cytogenetic Analysis of Patients with Primary Amenorrhea

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ABSTRACT Amenorrhea is a normal clinical feature in prepubertal, pregnant, and postmenopausal females. It also accounts for 20% of patients with infertility. The physiology of menstruation and reproduction has a strong correlation with the expression of the X chromosome. Thus, the role of genetics in terms of diagnosis, risk assessment, and genetic counseling is significant. The genetic contribution to amenorrhea is studied both at the cellular and molecular level aiming at abnormalities in chromosomes and mutations in genes. The present study aimed at performing chromosomal analysis in patients present with primary amenorrhea (n=140) employing GTG banding. The karyotype results revealed 71.2% (n=101) with normal chromosome composition and 27.8% (n=39) showed chromosomal abnormalities. In patients with abnormal chromosome constituents, 74% (n=29) exhibit numerical aberration and 26% (n=10) showed structural abnormalities. The X-chromosome abnormality was observed in 49% of the subject population which is consistent with results of studies conducted in the past. Also, the involvement of Y chromosome and origin of marker chromosome was confirmed by FISH in four patients.

INTRODUCTION

Genetic disorders pose considerable health and economic burden on affected individuals, families, and the community as well. As more environmental diseases are successfully controlled those that are wholly or partly genetically determined are becoming more important. Hence, the search for genetic component is of the utmost importance for diagnosis, risk assessment and genetic counseling. The common indications for genetic analysis include advanced maternal age, developmental delay, family history of chromosomal abnormalities and fertility problems. Menstruation or fertility is one of the most prevalent indications for genetic referral in females. Amenorrhea is a normal feature in prepubertal, pregnant, and postmenopausal females and it accounts for 20% of patients with infertility. The differential diagnosis of amenorrhea is broad and can range from endocrine disorders, genetic abnormalities, psychological, environmental, and structural anomalies. It has been reported that the percentage of chromosomal abnormalities varies from 15.9% to 63.3% in patients with primary amenorrhea (Wong and Lam 2005; Joseph and Thomas 1982).

Since the introduction of chromosome

banding, karyotyping is one of the standard diagnostic procedures for identifying chromosomal aberrations involved in the disorder. The Department of Human Genetics, Sri Ramachandra University, Chennai has been approved by the Government of Tamil Nadu to perform prenatal and postnatal genetic diagnosis. A summary of results of cytogenetic analysis were carried out in patients referred (Department of Human Genetics) for amenorrhea has been detailed below. The chromosome analysis was performed by GTG banding. In addition, FISH technique with locus specific probes was performed to confirm diagnosis as obtained with conventional GTG banding.

MATERIALS AND METHODS

Study Subjects

The study subjects included patients referred with primary amenorrhea (n=140) for chromosomal analysis to the Department of Human Genetics, Sri Ramachandra University, Chennai, India. The cases were referred from different parts of South India between 1998 and 2006. The age group of the subjects ranged from 13 to 30 years. Detailed pedigree analysis and in depth clinical evaluation and clinical reports were obtained from all subjects. About 1ml of peripheral blood was collected from each patient in a heparinized container and used for cytogenetic analysis as detailed below.

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Chromosome Preparation and GTG- Banding

About 1 ml of blood was supplemented with 8 ml of RPMI medium, 2 ml of fetal bovine serum, 0.1 µg/ml of PHA (Phytohemagglutinin) and incubated at 37°C with 5% CO₂. After 66½ hour of incubation, ethidium bromide (1mg/ml) was added followed by the Colchicine (1mg/ml) at 67th hour and incubated for another 1.5 hour (Verma and Arvind 1995). The cells were then harvested by hypotonic treatment (20 minutes with 0.075M KCl at 37°C), fixed and washed thrice with Carnoy's fixative (methanol and acetic acid in a ratio of 3:1) and casted on clean pre chilled slides. Multiple slides were casted for each sample and used for chromosomal aberration analysis and Fluorescence *in situ* hybridization (FISH). The slides were exposed to Trypsin 20mg/ml for 20–30 seconds, stained with 10% Giemsa, air dried and mounted with coverslip using DPX. For each sample, 25 metaphases were analyzed and the karyotype was interpreted (Shaffer and Tommerup 2005), using applied imaging software. Whenever, the mosaic or abnormal karyotype was obtained, a more number of metaphases (100) was analyzed.

Fluorescence *in situ* Hybridization

A standard protocol as directed in the kit (Vysis) was followed for FISH. The slides with metaphase chromosomes prepared as per standard cytogenetic procedure and was dehydrated in 70%, 80% and 100% ethanol for 2 min each at room temperature and air dried. The locus specific probe (DNA probes wcp X and Y;

CEP X and Y) was mixed with hybridization buffer and deionized distilled water and applied to the slides. The metaphase chromosomes and the probes were co-denatured using Hybrite at 73°C for 3 minutes. The slides were sealed with coverslip using rubber cement and hybridization was carried out for 24 hours at 37°C. After 24 hours of hybridization, the coverslip was removed and the slides were rinsed in formamide wash solution (0.4X SSC/0.3%NP-40) at 45°C and the slides were air dried. After air drying, the slides were counterstained with DAPI (7.5 µl/slide), covered with coverslip, stored in dark prior to signal enumeration and observed under fluorescent microscope for appropriate signals.

RESULTS

The chromosomal analysis and the obtained karyotype for the patients are summarized in table 1. The karyotype results revealed 71.2% with normal chromosome composition (n=101) and 27.8% (n=39) showed chromosomal abnormalities (Fig. 1). In patients with abnormal chromosome constituents, 74% exhibit numerical aberrations (n=29) and 26% with structural aberrations (n=10) (Fig. 2).

Figure 3 shows the distribution of numerical and structural aberrations among the patients with abnormal chromosomal constituents. About 28.2% of them showed complete monosomy 45,X (n=11), 17.9% of cases with male karyotype (n=7) and 51.2% with mosaic genotype (n=20). In mosaic cases, the ratio of the monosomic/trisomic cells to normal cells varied from 4% to 95% and the cases were indexed from Cyto001 Cyto020

Table 1: Karyotype details of the subjects referred for cytogenetic analysis with primary amenorrhea. (n=140)

Chromosomal abnormalities	Karyotype	Number of cases
Normal Karyotype	46,XX	101 (72.1%)
Numerical Abnormality		
Monosomy X	45,X	11 (7.8%)
Sex reversal	46,XY	07 (5%)
Turners Mosaic	45,X/46,XX	07 (5%)
	45,X/46,XX/47,XXX	02 (1.4%)
Presence of XY constitution	46,XX/46,XY	01 (0.7%)
	45,X/46,XY	01 (0.7%)
Structural Abnormality		
Marker chromosome	45,X/46,X +mar	03 (4.2%)
Isochromosome	45,X/46,X,iXq	04 (4.9%)
Inversion	45,X(80%)/46,X,inv(Xq)(q21-22)(20%)	01 (0.7%)
Isodicentric	45,X/46,X,idic(X)(q25)	01 (0.7%)
X-Autosome Translocation		
Translocation between X;20	46,X,t(X;20)(q22;q11.2)	01 (0.7%)

Table 2: Variation of cell lines from monosomic/trisomic to normal seen in mosaic patients.

Code Number	Karyotype
Cyto001	45,X(23%)/46,XX(77%)
Cyto002	45,X(80%)/46,XX(20%)
Cyto003	45,X(15%)/46,XX(85%)
Cyto004	45,X(5%)/46,XX(95%)
Cyto005	45,X(25%)/46,XX(75%)
Cyto006	45,X(10 %)/46,XX(90%)
Cyto007	45,X(4%)/46,XX(96%)
Cyto008	45,X(80%)/46,X,inv(Xq)(q21-22)(20%)
Cyto009	45,X(20%)/47,XXX(80%)
Cyto010	45,X(10%)/46,XX/(85%)/47,XXX(5%)
Cyto011	46,XX(70%)/46,XY(30%)
Cyto012	45,X(76%)/46,X,dic(X)(q25)(24%)
Cyto013	45,X(35%)/46,X +mar(85%)*
Cyto014	45,X(60%)/46,X +mar(40%)*
Cyto015	45,X(70%)/46,X+mar(30%)*
Cyto016	45,X(57%)/46,XY(43%)*
Cyto017	45,X(33%)/46,X,iXq(67%)
Cyto018	46,iXq(100%)
Cyto019	45,X(92%)/46,iXq(8%)
Cyto020	45,X(76%)/46,X,iXq(24%)

*To confirm the presence of marker chromosome, FISH technique was performed and identified as a part of chromosome X.

*To confirm the presence of 45,X/46,XY, FISH technique was performed and confirmed the presence of mosaic was the same as that of GTG.

(Table 2). The two patients with association of chromosome Y, was confirmed by FISH (Fig. 4). Similarly, the presence of marker chromosome in 3 patients was confirmed as a part of chromosome X, using FISH (Fig. 5). Among 26% of cases involved structural aberrations within the sex chromosomes, one case showed translocation between X and an autosome (Fig. 6).

DISCUSSION

Primary amenorrhea is the complete absence of menstruation. Genetic factors have a causal effect/relation in addition to endocrine disturbances, constitutional, and environmental factors in primary amenorrhea. Several cytogenetic studies have been performed aimed at understanding the frequency and nature of chromosomal abnormalities in primary amenorrhea (Franks 1987; Hunter 2006). In the present study, 27.8% (39/140) of the patients referred for primary amenorrhea had chromosomal abnormalities. However, in an earlier study abnormal chromosomal constitution was reported to be 25.6% in Indian population (Anupam 2004) and 24.5% in

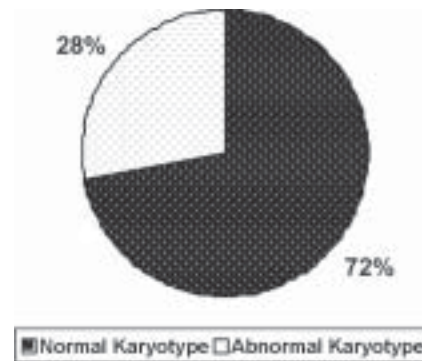


Fig. 1. The percentage of normal vs. abnormal karyotype in subjects referred for primary amenorrhea

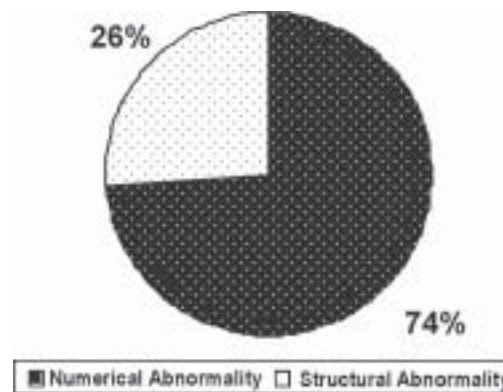


Fig. 2. The percentage of numerical and structural aberrations in subjects with abnormal chromosome constituents.

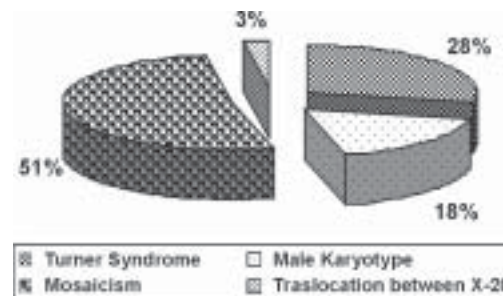


Fig. 3. The distribution of numerical and structural aberrations among the patients with chromosomal abnormalities.

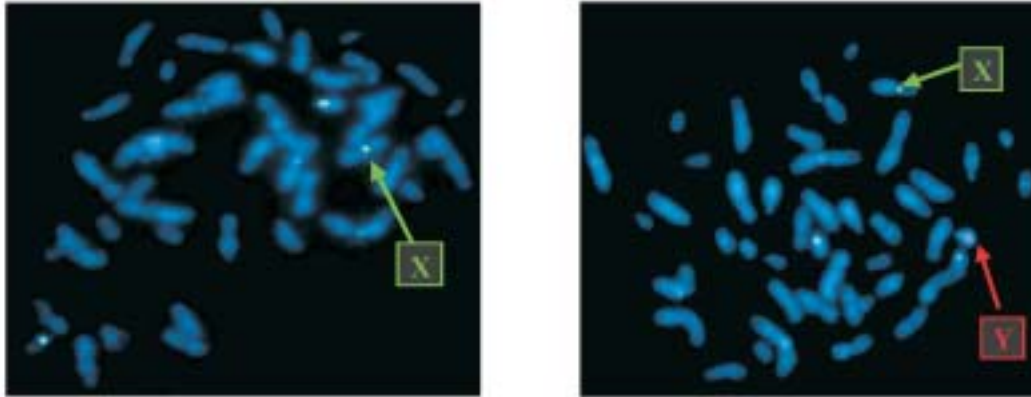


Fig. 4. Metaphase showing the presence of 45,X and 46,XY.

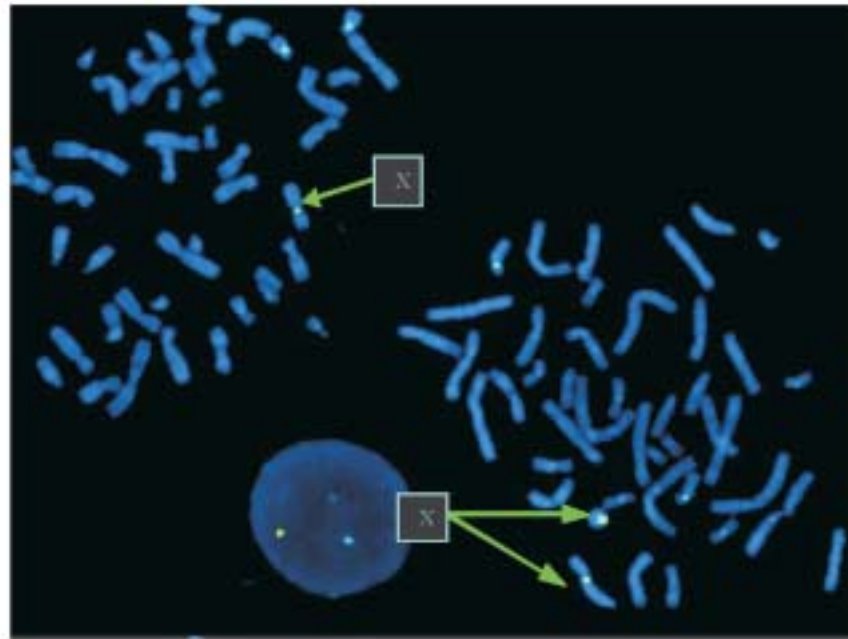
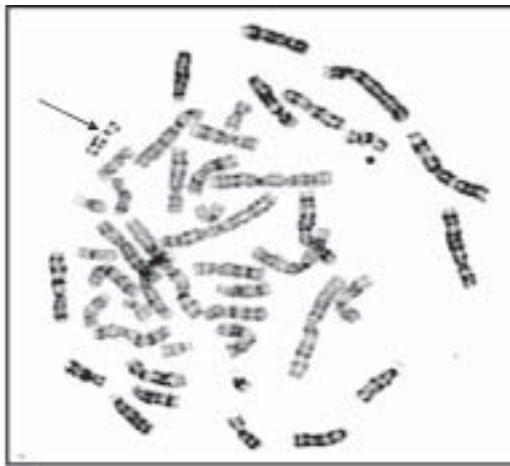


Fig. 5. Metaphase showing the presence of 45,X and the marker chromosome as X.

Chinese population (Wong and Lam 2005) among primary amenorrhea patients. The variation between the present and that of the previous studies may be due to the difference in the selection of patients included for analysis.

Among the different types of chromosomal abnormalities either complete monosomy of X (n=11) or in mosaicism (n=8) was observed in 49% of cases. The monosomy of X is the typical karyotype of Turner's syndrome. The obtained

results were in good correlation with that of published reports as Turner's syndrome is reported to be the leading cause of primary amenorrhea. The obtained results further strengthened the role of gene composition on the X-chromosome in the normal female physiology and reproduction. Due to lack of clinical data for some of the cases, we did not make any attempt to correlate the karyotype with physical features for all the cases. However, the short stature



in 18% (n=7) of the patients with primary amenorrhea. The obtained results are in agreement with the study that conducted in the past, which has reported 20% of cases with Y chromosome constitution referred for primary amenorrhea (Anupam 2004). Studies have demonstrated that a female karyotype can occur in XY embryo when testis determining factor (TDF) or other genes in the testes determining pathway are lost, mutated or compromised (Ostrer et al. 1989). A higher percentage (26.4%) of the females with primary amenorrhea with 46,XY karyotype has been reported and the importance of an accurate clinical and histological evaluation of patients with 45,X/46,XY mosaicism has been highlighted (Robert et al. 1987; Christine et al. 1986; Lewis 1970). Furthermore, when Y chromo-



Fig. 6. Metaphase and karyotype showing the presence X-autosome translocation

observed in majority of the cases with a 45,X karyotype appears to be triggered by the lack of a second sex chromosome in a small proportion of cells. Haploinsufficiency of the short-stature homeo box (SHOX) gene mapped to the pseudoautosomal region of the X and Y chromosomes has been causally responsible as reported earlier (Abir and Fisch 2001).

XY gonadal constitution in females was found

somal material is present in individuals with 45,X/46,XY mosaicism, there is 10 to 20% risk for developing gonadoblastoma (Lewis 1970). Hence, accurate detection of Y chromosome complement and their composition plays an important role in counseling. In the present study, we used Y-chromosome specific probe to accurately confirm the presence of Y chromosome in cases with sex reversal and mosaic condition.

The long term follow up study is intended to carry out in the forthcoming days.

It has been reported that primary amenorrhea patients with 45,X/46,X,mar(X) karyotypes may have usually severe phenotypes, which include mental retardation or abnormal facial features (Wolff 1996). Three cases in our study did not show any mental retardation, and it can be assumed that those cases contained XIC, thus no functional disomy. The molecular cytogenetic technique, FISH, accurately delineate the nature and origin of marker chromosomes, which is difficult by conventional cytogenetics.

While most of the studies in primary amenorrhea patients reported the involvement of sex chromosomes abnormality, we observed a translocation between X and an autosome (chromosome # 20). Two primary amenorrhea patients present with X-autosome translocation has been reported (Wong and Lam 2005). However, further study is needed to explain the role autosomal translocations with X chromosome and primary amenorrhea.

CONCLUSION

A significantly high number of primary amenorrhea patients had sex chromosomal abnormalities, suggest the urgent need of early accurate cytogenetic investigation to help the patient for a better counseling and management. After excluding the non-genetic causes by gynecologists, primary amenorrhea should receive prompt referral for genetic study.

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