



ORIGINAL ARTICLE

Frequency and pattern of cytogenetic alterations in primary amenorrhea cases of Kashmir, North India



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Abstract *Background:* Primary amenorrhea (PA) is proposed to have multiple etiological factors that include genetic factors, intrauterine malformations, endocrine dysfunction and environmental factors, as revealed by previous studies pertaining to amenorrhea. However, among the various proposed etiologies, genetic factors appear to be highly associated with PA as approximately 40% of PA cases have been found to have genetic causes.

Aim of the study: The present study was proposed to establish the frequency and pattern of chromosomal abnormalities in PA cases of Kashmir.

Subjects and methods: A total of 108 females within the age group of 14–33 years and having a history of amenorrhea were included in the study. Peripheral blood lymphocyte cultures were set for each subject according to standard protocol and chromosomal analysis was carried out on well spread metaphases by the help of Cytovision software Version 3.9.

Results: The results of the present study reveal that the incidence of chromosomal abnormalities in PA cases of this region is almost similar with those of many reports around the world. However, we report two unique chromosomal alterations viz., 46,XX, dup2q(13) and 46,XX, t(2,5)(p11.2;q34) that have not been found elsewhere in the literature.

Conclusion: The results of the present study indicate that chromosomal analysis of females with PA, after the exclusion of non-genetic causes, should be essentially considered for the precise diagnosis and the development of more successful treatment. The study being the first of its kind in this part of the world forms the basis for further studies of the PA cases of this region. The precise molecular characterization of the unique breakpoint regions reported in our study can possibly help in the identification of new genes involved in primary amenorrhea.

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1. Introduction

Primary amenorrhea (PA) is the absence of a menstrual period in a woman of reproductive age. Besides, it is defined as an absence of secondary sexual characteristics in a female who has attained an age of 14 but has not attained menarche or has normal secondary sexual characteristics but has not

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attained menarche by 16 years of age [1]. Amenorrhea is a symptom with many potential causes. PA may be caused by developmental problems such as the congenital absence of the uterus and failure of the ovary to receive or maintain egg cells. The main causes of PA include chromosomal or genetic abnormalities that can cause the ovaries to stop functioning normally, hypothalamic or pituitary problems in the brain and physical problems such as problems with reproductive organs that can prevent periods from starting and extreme physical or psychological stress, or a combination of these factors that can delay the onset of menstruation [2].

According to World Health Organization estimates, amenorrhea stands as sixth largest major cause of female infertility. Additionally, among general population amenorrhea affects 2–5% of all women in the child bearing age [3]. PA is primarily caused by pituitary/hypothalamic disorders (27.8%); gonadal dysfunction (50.4%) and outflow tract abnormalities (21.8%) [4]. However, the diagnosis disparity of amenorrhea is wide and can vary 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 [5,6]. Several cytogenetic studies have been undertaken to establish the frequency and type of chromosomal abnormalities in primary amenorrhea [7,8]. However, no study has been carried out in the primary amenorrhea cases of Kashmir, the Northern-most region of India. The present study was therefore, carried out with a purpose to unveil the type of chromosomal abnormalities and their frequency in 108 females with PA who were referred to our Center for cytogenetic evaluation.

2. Subjects and methods

2.1. Sample collection

The study subjects included patients with PA referred ($n = 108$) for chromosomal analysis to the Cytogenetic Laboratory of Advanced Center for Human Genetics, SKIMS, Srinagar-India from January 2013 to January 2015. The study was carried out in accordance with The Code of Ethics of the World Medical Association (Declaration of Helsinki) for experiments in humans. An informed consent was taken from each patient as per the norms of Institutional Ethics Committee before sample collection. The age group of the subjects ranged from 14 to 33 years with a mean of 21.7 ± 5.1 years. Pedigrees with details were drawn and in depth clinical evaluation and clinical information were obtained from all subjects. About 1–2 ml of heparinised peripheral venous blood sample was collected from each patient aseptically and each sample was given a unique laboratory number.

2.2. Setting up of lymphocyte cultures

Lymphocyte cultures were set according to Moorhead et al. [9]. Peripheral venous blood samples were aseptically transferred into sterile culture tubes with 5–8 ml of RPMI-1640 medium each (Sigma, Michigan, USA), supplemented with L-glutamine, 10% fetal bovine serum (Himedia Labs, India), Penicillin–streptomycin solution (Invitrogen, California,

USA) and phytohaemagglutinin (Himedia Labs, Mumbai, India). Parallel cultures were set for each sample. The culture tubes were marked accordingly and incubated in a CO₂ incubator for 72 h. 50 µl colchicine was added to each culture tube at the completion of 70 h to arrest the cells at metaphase. After 72 h of incubation, the cell suspensions were centrifuged for 10 min at 1000 rpm. The supernatant was discarded and the pellet was treated with hypotonic solution (0.075 M KCl) by gentle flushing and cyclomixed. The centrifuge tubes were incubated again at 37 °C for 45 min. The tubes were again centrifuged carefully at 1000 rpm for 15 min. The supernatant was removed and 5–8 ml of freshly prepared pre-chilled Cornoys fixative was added to the pellet while mixing on cyclomixer. The tubes were allowed to stand overnight and washed with freshly prepared pre-chilled Cornoys fixative repeatedly for 3–4 times.

2.3. Slide preparation and karyotyping

The slides were prepared by air-drop method and GTG banding was performed. The slides were stained with 1% Giemsa stain [10]. Karyotyping was performed with the help of Cytovision software Version 3.9 (Applied Imaging, Michigan, USA) on well spread G-banded metaphase plates. At least 30 metaphases were examined for each subject to rule out any chromosomal anomaly and mosaicism. Karyotypic reports were based on the International System for Human Cytogenetic Nomenclature recommendations, 2009.

3. Results

A total of 108 females with PA were evaluated for chromosomal abnormalities. Of the total number of females with amenorrhea, 70 (64.81%) had a normal karyotype (46,XX) while 38 (35.18%) had abnormal karyotype. The chromosomal abnormalities included both numerical as well as structural abnormalities. The numerical abnormalities observed in the patients were monosomy X (45,XO) (Fig. 1), mosaic monosomy X (46 XX/45 XO) and sex reversal (46,XY). Structural abnormalities observed include isochromosome (46,XX,iXq) (Fig. 2), deletion (46,XX,delXq23-qter) (Fig. 3), duplication (46 XX, dup2q31) (Fig. 4) and translocation 46,XX/46,XX,t(2;5)(p11.2;q34) (Fig. 5). The detailed karyotype particulars of the females, frequencies of different cytogenetic alterations, abdominopelvic ultrasonography findings and the hormonal profiles of the subjects are summarized in Table 1.

4. Discussion

Amenorrhea stands as one of the major causes of female infertility affecting 2–5% of all women of child bearing age [3]. Various conditions like intrauterine malformations, endocrine dysfunction and genetic factors form the etiological basis of PA. As revealed by previous studies pertaining to amenorrhea, approximately 40% of PA cases have been found to have genetic causes [11]. These can be single gene disorders or chromosomal disorders. However, chromosomal abnormalities contribute to the constitutional etiology of amenorrhea [12].

Several studies have been carried out to determine the frequency of chromosomal abnormalities among patients with

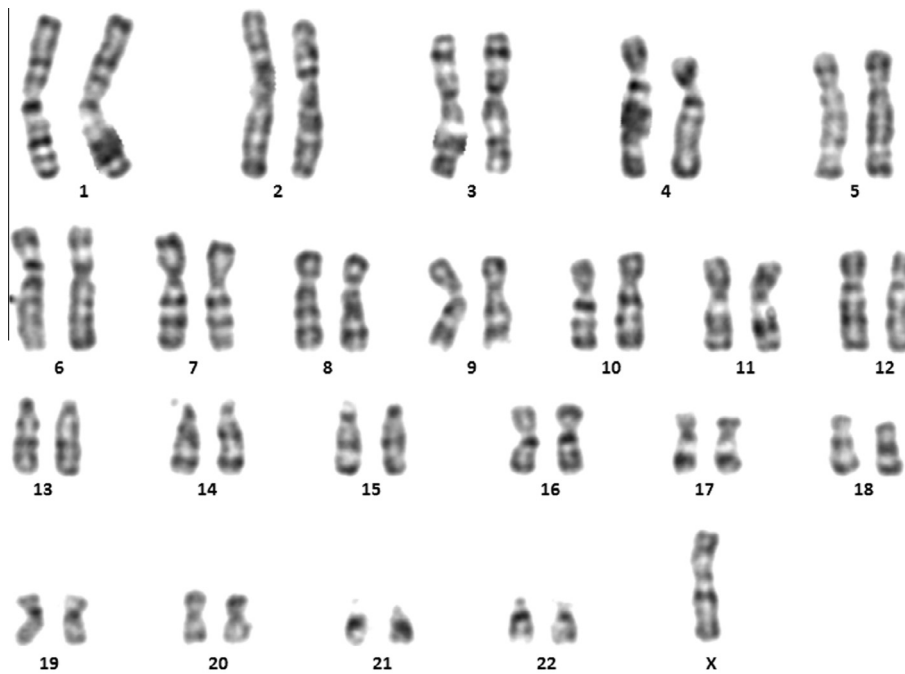


Figure 1 Representative karyotype (46,XO) of a primary amenorrhea female with Turner's syndrome.



Figure 2 Representative karyotype (46,XX,i(Xq)) of a primary amenorrhea female with isochromosome Xq.

primary amenorrhea [13–16]. In a survey on a large sample size of Andhra Pradesh, India ($n = 944$), Jyothy et al. identified 21.5% of women with PA to have an abnormal karyotype [17]. However, the literature suggests that no study on cytogenetic analysis of PA cases has been carried out in Kashmir population (North India) and ours is the first report on chromosomal abnormalities in PA females of this region.

Among the PA cases referred to our Center, 35.18% had one or the other chromosomal abnormality. However, evidence suggests that chromosomal abnormalities are present in 46–62% of patients with primary amenorrhea and the abnormalities include X chromosome aneuploidy, XY karyotype, isochromosome X, isodicentric, rings, and deleted or inverted X chromosomes [18]. In an earlier study abnormal

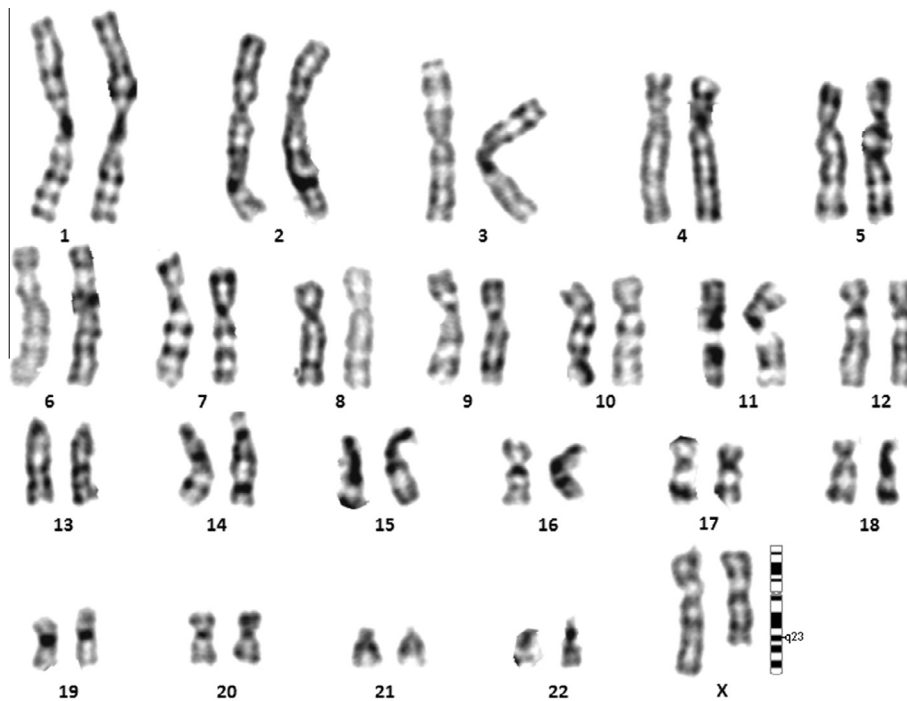


Figure 3 Representative karyotype [46,XX,del(Xq23-qter)] of a primary amenorrhea female with deletion Xq.

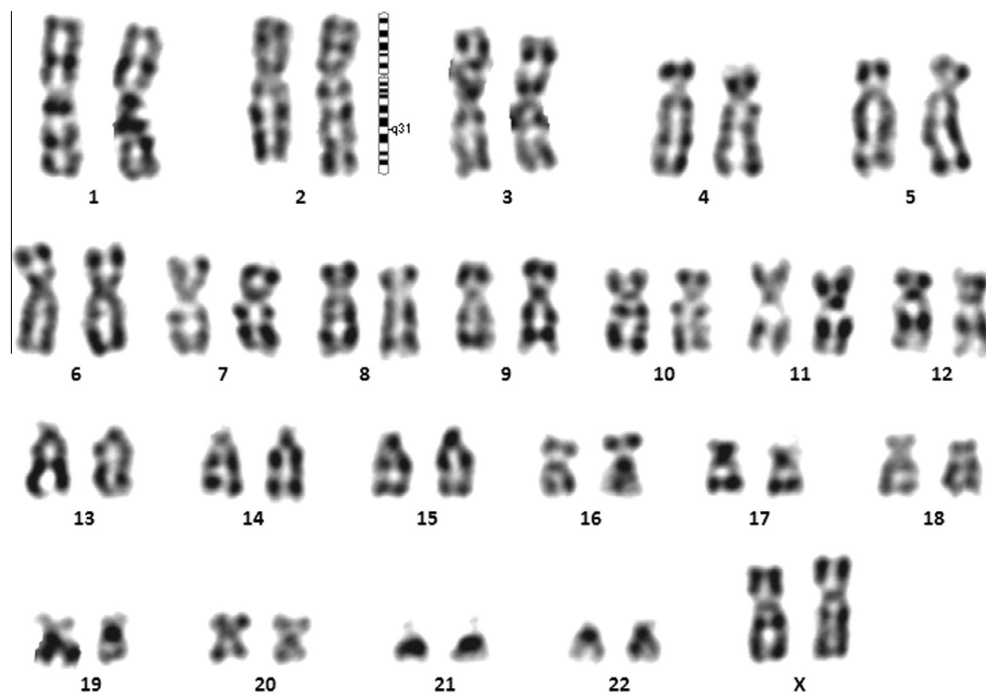


Figure 4 Representative karyotype [46,XX,dup(2q31)] of a primary amenorrhea female with duplication 2q.

chromosomal component was reported to be 25.6% in Indian population [19] and 24.5% in Chinese population [4] among PA patients. The variation in the frequency of chromosomal abnormalities in these studies can be attributed to the difference in the number of subjects and their selection criteria.

The abnormalities observed in the subjects of our study were complete monosomy X (Turner's syndrome), mosaic monosomy X (Mosaic Turner's syndrome), isochromosome Xq (Variant Turners syndrome), 46,XY (male karyotype), deletion (Xq) and duplication (2q). Turner's syndrome

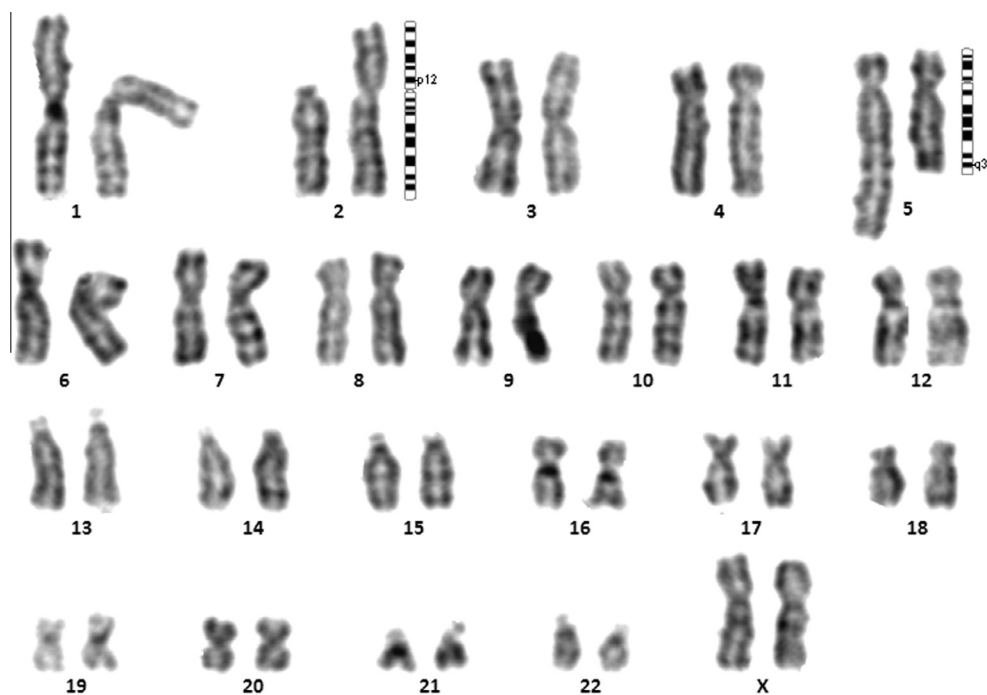


Figure 5 Representative karyotype [46,XX/46,XX,t(2;5)(p11.2;q34)] of a primary amenorrhea female with translocation (2;5) chromosomes.

| Cytogenetic grade | Karyotype | No. of cases | Frequency (%) | Hormonal status | Ultrasonography findings (No.) |
|-------------------------------|-----------------------------------|--------------|---------------|--|---|
| <i>Normal karyotype</i> | 46,XX | 70 | 64.81 | Increased FSH/LH | Normal (22), Mullerian agenesis (12), Hypoplastic uterus (8), Streak ovaries (17), pre-pubertal uterus (11) |
| <i>Numerical abnormality</i> | | | | | |
| Monosomy X | 45,XO | 26 | 24.07 | Increased FSH/LH, Decreased estradiol | Small atrophied uterus (8), Streak gonads (11), Absent ovaries (7) |
| Sex reversal | 46,XY | 3 | 2.77 | Increased FSH (2), Increased Testo (1) | Streak gonads (1), rudimentary/absent uterus (1), Inguinal hernia (1) |
| Turners mosaic | 45,XO/46,XX | 5 | 4.62 | Increased FSH/LH | Hypoplastic uterus (3), Small ovaries (2) |
| <i>Structural abnormality</i> | | | | | |
| Isochromosome | 46,XX,i(Xq) | 1 | 0.92 | Increased FSH | Streak ovaries, Infantile uterus |
| Duplication | 46,XX,dup(2q31) | 1 | 0.92 | Increased FSH/LH | Normal findings |
| Deletion | 46,XX,del(Xq23-qter) | 1 | 0.92 | Increased FSH | Hypoplastic uterus, ovaries not visualized |
| Translocation | 46,XX/ 46,XX,t(2;5)(p11.2;q34) | 1 | 0.92 | Increased FSH/LH | Streak like ovaries, infantile uterus |

(complete or mosaic monosomy X) is reported to be the leading cause of primary amenorrhea as per the published reports [13] and our results are in good correlation with them as 28.69% of the subjects of our study proved to be Turner syndromes.

A significant percentage (2.77%) of patients with primary amenorrhea presented with a male karyotype. This could be due to the conditions like sex reversal or androgen insensitivity. All these females appeared tall for their age. Among these,

two patients presented to a gynecologist when they were over the age of 30 years and were married. This emphasizes the importance of an early cytogenetic diagnosis of these females so that they could be managed at a proper time.

Deletions involving the long arm of X chromosome generally results in ovarian failure if they involve the proposed critical region Xq13-q26 [20]. This explains the cause of one of our patients with deletion of Xq (Xq23-qter) who had primary amenorrhea, but no features of Turner's syndrome.

One of the females was found to have 46,X,i(Xq) karyotype. She had all features of Turner syndrome. Structural abnormalities leading to PA mostly are isochromosome of the long arm of X [i(Xq)] [21]. The isochromosome (Xq) appears as a metacentric chromosome that has isologous arms, which are structurally identical and contain the same genes. This leads to the partial monosomy of the genes present on Xp and partial trisomy of genes present on Xq. Females with 46,X,i(Xq) karyotype have been found to manifest streak gonads. Complete ovarian failure and partial ovarian failure have also been reported in 91% and 9% of cases with i(Xq) individuals. Besides, short stature and TS stigmata are found to be frequent with almost complete lack of gonadal development in 46,X,i(Xq) females. In contrast to this, 50% of females with 46,XX,del(Xp11) karyotype menstruate or develop breasts [22].

Duplication of chromosome was observed in one of the females with a karyotype 46,XX,dup(2q31). In general, duplications are less harmful than deletions but they inevitably are associated with some clinical abnormalities. The degree of clinical severity is correlated with the size of the duplicated segment. Duplication results in partial trisomy of the chromosomal segment that is duplicated and trisomy of the genes present in that particular segment. In primary amenorrhea cases Xp/q chromosome duplications have been reported by many studies [3,23]. However, evidence suggests that duplication 2q31 has not been reported so far in primary amenorrhea cases making it a unique one.

Translocation (2;5)(p11.2;q34) is another unique chromosomal alteration observed in one of the subjects of our study. The female was found to be mosaic with karyotype 46,X X/46,XX,t(2;5)(p11.2;q34). Ultrasonography report of the patient revealed infantile uterus with bilateral streak ovaries. While many of them have reported X-autosome translocations [13,4,24] in PA cases, several other studies at isolated places have reported autosome-autosome translocations [3,25]. Besides, Tupler et al. have reported two unrelated women with gonadal dysgenesis who were found to have balanced autosomal translocations i.e. t(6;15)(p21.3;q15) and t(8;9)(p11.2;q12), respectively [26]. The enigma of gonadal dysgenesis in such cases could possibly be attributed to the presence of autosomal gene(s) playing a role in the normal gonadal development [27].

5. Conclusion

A significant percentage of the cases of the present study was found to have chromosomal abnormalities as a plausible cause of PA. This indicates that chromosomal analysis of females with PA, after the exclusion of non-genetic causes, should be essentially considered for the precise diagnosis and development of more successful treatment. Moreover, ascertainment of the karyotyping aids in genotype correlation to understand clinical heterogeneity, and in genetic counseling. Among the novel karyotypes, we report for the first time two unique cases of chromosomal alterations associated with PA. Besides, precise molecular characterization of these chromosomal alterations could help in identification of new genes or genes involved in PA and also help in better understanding of molecular mechanism underlying the aberrations. The study being the first of its kind in this part of the world forms the basis for further studies of PA patients of this region.

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