Original Article

Deoxyribonucleic acid damage study in primary amenorrhea by comet assay and karyotyping

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AIM: This study aims at evaluating the chromosomal abnormalities and deoxyribonucleic acid (DNA) damage in cases with primary amenorrhea by karyotyping and comet assay.
STUDY DESIGN: A total of 30 cases of primary amenorrhea were recruited. Secondary sexual characters were assessed by Tanner staging. Chromosomal analysis was performed by conventional phytohemagglutinin stimulated lymphocyte cell culture technique. Alkaline version of comet assay was used to evaluate DNA damage.
RESULTS: The chromosomal pattern of 20 subjects (66.7%) was found to be normal (46,XX). Two subjects had 46,XY pattern and eight subjects had Turner syndrome (45,X or 45,X/46,XX). The comet parameters were found to be increased among subjects with 45,X monosomy, when compared to the rest of the study group and also in subjects with Tanner stage 1 when compared to stage 2.
CONCLUSION: Comet assay revealed increased DNA damage in cases with 45,X monosomy, compared with subjects with 46,XX and 46,XY karyotype, which correlated with clinical features.

Key words: Comet assay, deoxyribonucleic acid damage, karyotyping, primary amenorrhea

Introduction

Primary amenorrhea is defined as the absence of secondary sexual characters or absence of menstruation by the age of 14 and 16 respectively.[1,2] According to Federation of Obstetrical and Gynecological Society of India, the incidence of primary amenorrhea in the Indian population is reported to be 2.5%. There are various etiologies of primary amenorrhea, which include congenital developmental abnormalities, genetic, metabolic, endocrine and infectious causes. Out of these, chromosomal abnormalities alone account for 43% of cases.[3]

Cytogenetic studies play a significant role in diagnosing clinically suspected primary amenorrhea cases. Out of the various studies, conventional karyotyping is the most commonly used method.[4] Oxidative deoxyribonucleic acid (DNA) damage has been attributed to be the cause of development of these abnormalities. Comet assay is a rapid, non-invasive, sensitive, inexpensive technique for quantification of DNA damage.[5] Alkaline version of comet assay is the widely accepted method.[5,6] The present study is undertaken to study the level of DNA damage using comet assay in primary amenorrhea subjects and to correlate the level of DNA damage with chromosomal aberrations studied by karyotyping and clinical features.

Access this article online	
Quick Response Code:	Website: www.ijhg.com
	DOI: 10.4103/0971-6866.124363

Materials and Methods

The present study is a descriptive study conducted in the Cytogenetic division of Department of Anatomy, from January 2010 to May 2011. After obtaining approval from the Institute Ethics Committee, 30 clinically diagnosed subjects with primary amenorrhea were recruited from the Obstetrics and Gynaecology outpatient wing of Jawaharlal Institute of Postgraduate Medical Education and Research. After getting their written informed consent,

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they were subjected to careful clinical examination, to look out for secondary sexual characters and any other associated abnormalities. Subjects with imperforate hymen and those exposed to ionizing radiation and pesticides were excluded from the study. Ultrasound examination and hormonal studies were done.

Chromosomal analysis (conventional karyotyping)

Lymphocytes were grown in culture media containing RPMI-1640 (HIMEDIA) with L-Glutamine (0.5 mg/ml), Phytohemagglutinin-m, fetal bovine serum (10%) and antibiotics for 72 h at 37.5°C. Cell culture was harvested using colchicine as mitotic inhibitor and KCl as a hypotonic solution.[4] Chromosomal preparation was stained with GTG banding. Metaphase spreads were captured and analyzed using IKAROS Metasystem software-Karl Ziess, Germany.

DNA damage analysis (comet assay)

Lymphocytes were separated by centrifuge using Histopaque and sandwiched between agarose gel layers and subjected to the lysis buffer (NaCl, TRIS, ethylenediaminetetraacetic acid and TITRON-X). Treatment of agarose-embedded cells with hypertonic lysis solution and non-ionic detergent removes their cell membranes, cytoplasm and nucleoplasm and dissolves nucleosomes. Subsequently, when the leftover nucleotide is treated with high alkaline solution, DNA supercoils unwind, thereby exposing the alkali labile sites (apurinic/apyrimidinec sites), which appear as breaks. Such breaks migrate towards the anode when exposed to current during electrophoresis thereby producing a "comet"-like appearance. Later slides were stained with silver nitrate. For screening the slides, a bright field light microscope Olympus BX, were used. Randomly 50 cells were selected under 20× magnification. The captured images were analyzed using Comet score software. Comet metrics such as comet length, head diameter, tail length, %DNA in the tail and %DNA in the head were studied.

Statistical analysis

Secondary sexual characters were staged using Tanner staging. Subjects were divided into two groups based on Tanner staging. The comet parameters were compared between them using Student's unpaired *t*-test. Based on chromosomal analysis subjects were divided into two groups, the first group with normal chromosomal complement and the second group with Turner karyotype. The comet parameters were compared between the two groups using Student's unpaired *t*-test.

Results

Subjects recruited for the study, were in the age group of 14-25 years. The mean height of subjects with a normal chromosomal pattern was 151.4 ± 7.88 cm, while that of subjects with an abnormal chromosomal pattern was 132 ± 6.45 cm [Table 1]. All the eight subjects with 45, X monosomy were short stature with height <145 cm [Table 1].

Clinical features

Clinical examination was done in all subjects. Among the 20 subjects with normal karyotype, two subjects had Mullerian agenesis. Four subjects had associated clinical features. One subject had small ears (microtia), hemivertebrae and renal agenesis, while the other three subjects had megaloblastic anemia, systemic lupus erythematosus and tuberculosis infection, respectively. Four subjects had weight less than the expected range for their age. The rest of the subjects had no obvious clinical abnormalities except for poorly developed secondary sexual characters. The common clinical features observed in girls with abnormal chromosomal pattern were shown in Table 2.

Tanner staging

The secondary sexual characters were evaluated on the basis of Tanner staging [Table 3]. All the subjects with normal karyotype (46,XX) ($n = 20$) who participated in the study, had poor development of secondary sexual characters, except for two, who had Mullerian agenesis,

Table 1: Distribution of subjects according to height (n=30)

Height (cm)	Number of subjects	Percentage
<145		
46,XX	5	43.3
45,X	8	
>145		
46,XX	15	56.7
46,XY	2	

presented with normal secondary sexual characters. One of the subjects with 46,XY (n = 2) pattern had well developed breasts (Tanner stage 3), absent axillary and pubic hair with blind vagina, while the other had poorly developed breasts, absent axillary and pubic hair with ambiguous genitalia. All the subjects with Turner karyotype (n = 8), showed typical Turner features and poorly developed secondary sexual characters.

Ultrasonographic findings

USG examination of all subjects with Turner karyotype (n = 8), revealed the presence of an infantile hypoplastic uterus with streak ovaries [Table 4]. USG examination in two individuals with 46,XY showed absent of the uterus and ovaries. Testis was present in left inguinal region in one of them and in the region of labia majora in the other.

Hormonal profile

Hormonal profile of two subjects with Mullerian agenesis was normal, indicating the presence of normal ovaries. Two subjects with 46,XY chromosomal pattern, showed an increased level of testosterone. Subjects with Turner karyotype, revealed increased follicle-stimulating hormone, with normal levels of all the other hormones, indicating the presence of streak ovaries. All the other subjects with 46,XX showed normal hormonal profile.

Chromosomal analysis

20 (66.7%) out of the 30 members in the study, had a normal chromosomal pattern (46,XX). Two of these 20 subjects had Mullerian agenesis (46,XX), with absent uterus and normal ovaries. Two subjects (6.7%) presented with 46,XY chromosome constitution (male pattern) with increased levels of testosterone. Both of them had androgen insensitivity syndrome, an X-linked recessive disorder. Eight subjects (26.6%) were found to have Turner syndrome [Figure 1a and b]. Four of them had pure 45,X chromosomal complement and the remaining four had mosaic pattern 45,X/46,XX. One out of the 45,X complement, presented with typical features of Kabuki syndrome (make up syndrome), which is a variant of Turner syndrome. One of the subjects with 45,X had spontaneous puberty at the age of 18. This is supported by the previous study by Pasquino *et al.*, which reported incidence of 3-15% of spontaneous puberty in Turner syndrome individuals.[7]

Table 2: Clinical features of subjects with abnormal chromosomal pattern (Turner karyotype)

Clinical features	45, X karyotype (n=4)	45,X/46,XX mosaic (n=4)
Short stature	4	4
Sexual infantilism	4	4
Low posterior hairline	3	2
Shield like chest	4	4
Wide spaced nipples	4	4
Cubitus valgus	4	4
Webbed neck	4	2
Short 4th metatarsal	4	2
Mental retardation	2	1
High arched palate	3	1
Micrognathia	1	0
CVS abnormality	2	1
Spontaneous puberty	1	0

CVS: Cardiovascular system

Table 3: Distribution of subjects according to the development of secondary sexual characters (n=30)

Tanner staging	Number of patients	Percentage
Breast		
1	11	36.6
2	18	60
3	1	3.3
Pubic hair		
1	20	66.7
2	10	33.3

Table 4: Distribution of subjects according to USG findings (n=30)

Site	USG findings	Number of patients
Uterus	Absent	7
	Aplastic	1
	Hypoplastic	17
	Bicornuate	1
	Normal	4
Ovaries	Absent	8
	Streak	8
	Small	3
	Polycystic	8
	Normal	1
Testis	Inguinal testis	1
	Testis in labia majora	1

USG: Ultrasound sonography

Comet analysis

Comet parameters were analyzed using Comet Score software. Comet length, tail length and head diameter showed statistically significant increased levels among subjects with abnormal chromosomal pattern (45,X,45,X/46,XX) when compared to the rest of the study group with chromosomal pattern 46,XX and 46, XY [Table 5, Figures 2 and 3]. Comet length (63.1 μm) and tail length (9.45 μm) were decreased in the subject who had spontaneous puberty as compared

to other Turner subjects (Comet length - 80 μm and tail length-25.4 μm), which may explain the reason.

The comet parameters were compared with development of secondary sexual characters graded using Tanner staging [Table 6]. Subjects with Tanner stage 1 showed increased comet parameters than in subjects with Tanner stage 2. From the comparison, it is clearly evident that the length of the comet was directly proportional to the extent of DNA damage.

Discussion

Based on the results of chromosomal analysis, subjects were categorized into two groups. First group comprising of individuals with 45,X monosomy, 45,X/46,XX and the second group comprising of individuals with 46,XX and 46,XY. The chromosomal analysis revealed normal chromosomal pattern in 20 subjects (66.7%) and sex chromosomal abnormalities in 10 subjects (33.3%), which included seven subjects with Turner syndrome, one subject with Kabuki syndrome[8,9] and two with androgen insensitivity syndrome. The appearance of mosaic cell lines (45,X/46,XX) ($n = 4$) in the present study may be explained by the occurrence of non-disjunction and anaphase lag in the zygote.

The integrity of a critical area (Xq13q26) in the X chromosome is essential for normal ovarian function.[10,11] A majority of the genes responsible for the development of secondary sexual characters and clinical features are localized in the short arm of the X chromosome, consistent with the findings, that most of the Turner phenotype appears to result from a reduced dosage

Table 5: Comparison between normal karyotype and Turner karyotype

Parameters	Turner karyotype (*n*=8)	Normal karyotype (*n*=22)
Comet length (μm)	80.45±14.61	52.43±6.90***
Head diameter (μm)	55.18±12.56	38.77±5.25***
% DNA in head	86.77±7.48	86.00±8.31
Tail length (μm)	25.40±14.77	13.60±7.94**
% DNA in tail	12.70±7.53	14.00±8.31

*P<0.05, **P<0.01, ***P<0.001, DNA: Deoxyribonucleic acid

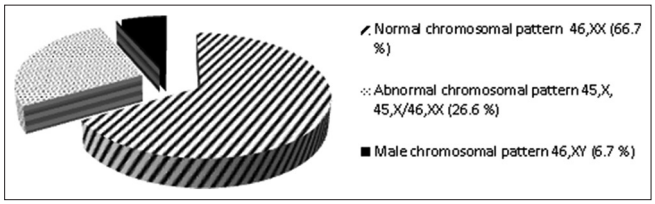

Figure 1a: Distribution of subjects according to chromosomal analysis

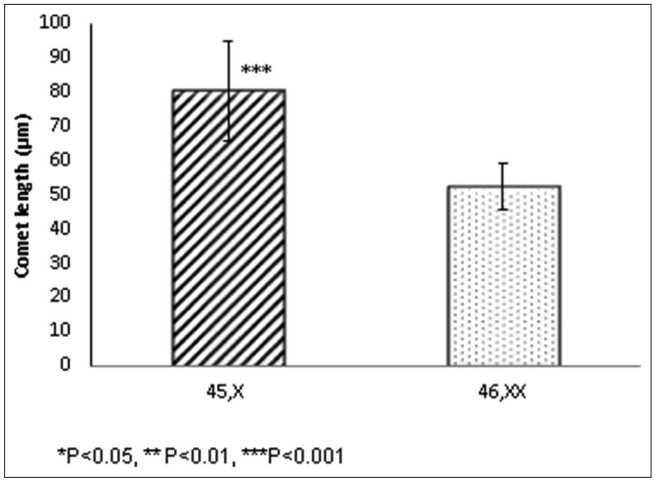

Figure 1b: Outcome of 30 subjects with primary amenorrhea

Figure 2: Comparison of comet length (μm) between 45,X (Turner karyotype) and 46, XX (Normal karyotype)

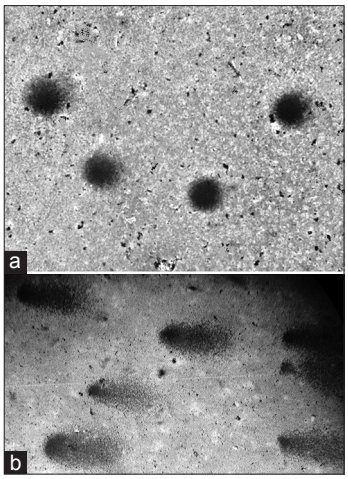

Figure 3: Comparison of comet between 46,XX (normal karyotype), and 45,X (Turner syndrome)

Table 6: Comparison of comet parameters between subjects with breast Tanner stage 1 and 2

Parameters	Tanner stage 1	Tanner stage 2
Comet length (μm)	62.70±19.97	58.47±13.19
Head diameter (μm)	47.94±11.61	40.54±9.40
% DNA in head	89.03±4.35	84.64±9.43
Tail length (μm)	14.85±13.32	17.86±10.38
% DNA in tail	10.58±4.19	15.35±9.44

DNA: Deoxyribonucleic acid

of genes on Xp (short arm of the X chromosome).[12] Ovarian failure in subjects with primary amenorrhea may be due to the DNA damage in the critical area of the X chromosome.

Comet analysis

Previous studies have reported DNA damage to be the primary cause of chromosomal aberrations.[13-15] Literature search does not reveal studies correlating the clinical features of primary amenorrhea with that of the DNA damage. Study done by Husain and Bamezai in 20 cases of primary amenorrhea using sister chromatid exchange, failed to correlate the DNA damage with chromosomal abnormalities.[16] In the present study, significant positive correlations between comet tail length and the chromosomal aberrations was observed. Probably, the present study might be the first study to correlate DNA damage and chromosomal aberrations with clinical features in cases with primary amenorrhea, in Indian population. In the current study, comets with increased tail length were observed in cases of Turner syndrome, suggestive of increased levels of DNA damage attributed to impaired DNA repair mechanisms, which in turn lead to the occurrence of chromosomal aberrations.[14] In comparison with subjects with 46,XX, the comet length values were significantly higher among the Turner syndrome individuals (45,X monosomy, 45,X/46,XX: 80.5 ± 14.6 and 46,XX, 46,XY: 52.4 ± 6.9, $P \leq 0.001$). This increase in comet length is not only because of a significant increase in comet tail length (45,X monosomy, 45,X/46,XX: 25.4 ± 14.8 and 46,XX,46,XY: 13.6 ± 7.9, $P \leq 0.01$) but also significant difference in Head diameter (45,X monosomy, 45,X/46,XX: 55.2 ± 12.6 and 46,XX, 46,XY: 38.8 ± 5.3, $P \leq 0.001$). Though the DNA damage values were found to be higher in both groups indicating oxidative DNA damage, due to generation of free radicals, the tail length and other parameters were increased more in Turner karyotype, which can be explained by the fact that Turner syndrome individuals presented with numerous chromosomal aberrations. From the comparison, it is clearly evident that the length of the comet is proportional to the extent of DNA damage. This correlated well with the previous study done by Husain and Bamezai using sister chromatid exchange in cases with primary amenorrhea.[16]

From the present study, we conclude that, DNA damage was more in individuals with chromosomal aberrations and more in subjects with Tanner stage 1 than in Tanner stage 2. DNA damage correlated with poor development of secondary sexual characters. In our study we have correlated the genetic factors that cause primary amenorrhea and its relevance in clinical features. Since the sample size in our study was small, future studies can be done by increasing the sample size and by performing biochemical profile of oxidative stress parameters, since oxidative stress is the prime cause for DNA damage.

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Cite this article as: Ramamurthy S, Chand P, Chaturvedula L, Rao KR. Deoxyribonucleic acid damage study in primary amenorrhea by comet assay and karyotyping. Indian J Hum Genet 2013;19:397-402.

Source of Support: Nil, **Conflict of Interest:** None declared.