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Genetics

ANALYSIS BY DNA OLIGO-ARRAYS MOLECULAR CYTOGENETIC IDENTIFIED A SIZE OF 40 MB RING X CHROMOSOME 46/45 MOSAIC GIRL SUSPECTED FOR TURNER SYNDROME

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ABSTRACT **Purpose:** A number of people meet with the infertility in their reproductive ages. If we list out the reasons of infertility state there are a number of problems. Among these problems, primary amenorrhea is a condition that leads to many other diseases/symptoms. Ring chromosome of X chromosome is one of the structural anomalies which are associated with manifestation of primary amenorrhea due ovarian dysgenesis. Ring chromosome may be present in mosaic form or present in all cells of an individual. **Methods:** Clinical history of 14 years girl was recorded. Around 5 ml peripheral blood was drawn in a sterilized syringe under complete aseptic condition. Whole blood culture was set with 0.5 ml peripheral blood for cytogenetic experiments like G banding, Karyotyping including FISH. Rest of the peripheral blood was used for extraction of genomic DNA for DNA HD-oligo array. **Results:** Analysis by DNA oligo-arrays molecular cytogenetic identified a size of 40 Mb ring X chromosome 46/45 mosaic girl suspected for Turner Syndrome **Conclusion:** It was concluded that ring (X) chromosomes lead to phenotypic heterogeneity which varies remarkably with its gene content and level of mosaicism.

KEYWORDS : Karyotyping, Mosaic ring X, Chromosome X, Variant Turner Syndrome, DNA HD-oligo array, Primary Amenorrhea

INTRODUCTION

A number of people meet with the infertility in their reproductive ages. If we list out the reasons of infertility state there are a number of problems. Among these problems, amenorrhea is a condition that leads to many other diseases/symptoms. World Health Organization estimates, out of 15% fertility, amenorrhea stands as sixth largest major cause of female infertility. Apart from that among general population amenorrhea affects 2–5% of all women in the child bearing age. Amenorrhea can be categorised into three major groups as Primary amenorrhea (PA), Secondary amenorrhea (SA) and Oligoamenorrhea.

Primary amenorrhea is defined as the absence of menstrual cycle by the age of 14 with no development of secondary sexual characteristics or the absence of menses by age of 16 with normal development of secondary sexual characteristic.

Amenorrhea stands as sixth largest major cause of female infertility according to the WHO. 15.9% to 63.3 % chromosomal abnormalities have been reported with Primary amenorrhea. Chromosomal abnormalities varied form numerical to structural abnormalities in PA. Ring chromosome of X chromosome is one of the structural anomalies which are associated with manifestation of primary amenorrhea due ovarian dysgenesis. Ring chromosome may be present in mosaic form or present in all cells of an individual. Ring chromosomes are formed by either terminal breaks of two arm of the chromosome and rejoining leading to loss of genetic material (Singurdardottir et al., 1997), or due to telomere-telomere fusion with no deletion, resulting in to complete ring (Guilherme et al., 2013) so-called Mc Clintock mechanism (Baldwin et al., 2008). Other complex mechanism of its formation includes terminal deletion and a contiguous inverted duplication due to an inv-dup-del rearrangement (Seghezzi et al., 1999; Knijnenburg et al., 2007).

Phenotype associated ring X chromosome are common features of TS which include short stature, peripheral edema, characteristic facial features, low posterior hairline, ovarian dysgenesis, endocrine disorders, and autoimmune conditions. Besides these, individual with r(X) are presented with mental disorders, learning difficulties, autistic spectrum disorders, craniofacial abnormalities, cardiovascular problems, skeletal system problem and skin related problems in severe condition (Denniset al., 2000; Leppig et al., 2004; Van Dyke et al., 1992; Lindgren et al., 1992; Prandstraller et al., 1999). Phenotype associated with ring chromosome is highly variable, because in addition to variability in loss genetic material during ring chromosome formation, secondary loss or gain also occurs due to ring chromosome instability (Tumer et al., 2004; Purandare et al., 2005; Glass et al., 2006). Clinical manifestation in these cases is dependent on origin, size, replication timing of the ring chromosome, genes affected by copy number variations, level of mosaicism and status of X inactivation.

Deletion of XIST at Xq13 results in to severe consequences due to failure of inactivation of X chromosome (Dennis et al., 2000). Extent of genetic material loss from Xqter towards the centromere is directly associated with incidence and severity of ovarian dysgenesis (Eggermann et al., 2005). Conversely, most of the patients with primary amenorrhea or gonadal dysgenesis had large Xq deletion as compared to patients with secondary amenorrhea or normal pubertal development (Adamson et al., 2002; del Rey et al., 2010; Leppig et al., 1993; Ogata et al., 2010; Tachdjjan et al., 2008; Mercer et al., 2013; Kim et al., 2014). Cytogenetic characterization in premature ovarian follicle cases suggested deletion in two distinct critical regions (CRs): CR1 and CR2 involving Xq13-q21 and Xq23-q28 respectively (Toniolo et al., 2006).

Case referred from the Sir Sunder Lal Hospital, Banaras Hindu University, Varanasi, India, were registered at our centre, Centre for Genetic Disorders, Banaras Hindu University, Varanasi. Parents had given their written consent for the detailed study. Clinical history was recorded. Around 5 ml peripheral blood was drawn in a sterilized syringe under complete aseptic condition. Whole blood culture was set with 0.5 ml peripheral blood for cytogenetic experiment and rest of the peripheral blood was used for extraction of genomic DNA.

MATERIALS AND METHODS

Whole blood culture and Karyotyping

Whole blood culture was set in 5ml RPMI-1640 pH 7.2 (Sigma-Aldrich, Inc., St. Louis, MI, USA) culture media supplemented with 10% fetal bovine serum (Himedia, India), 50 µg/ml of antibiotic (Gentamycine) and induced by phytohaemagglutinin-M (Sigma-Aldrich, Inc., St. Louis, MI, USA). Culture, staining and karyotyping was performed according to the procedure described.

Fluorescence in situ hybridization (FISH)

Fluorescence in situ hybridization (FISH) is a molecular cytogenetic technique that uses fluorescent probes that bind to only those parts of the chromosome with a high degree of sequence complementarity. It was developed in early 1980s and used in localization of some DNA sequence on chromosome for genetic counselling and medicine. Fluorescence in situ hybridization (FISH) was performed using probe for peri-centromeric region of chromosome X [CEP X (DXZ1)] (Abott vysis) labelled with Spectrum Green. 1 µl of probe, 7 µl of hybridization buffer and 2 µl nuclease free water was used for each slide hybridization. Probe was denatured by incubation at 73 0C for 5 minutes. The probe was kept at 40-450C until ready to apply probe to target DNA. Slides with chromosome were denatured at 730C for 5 minutes with 70% formamide in 2XSSC solution. Slides were serially dehydrated with 70%, 85% and 100% alcohol and transferred to hot plate maintained at 45 0C. 10 µl of denatured probe was hybridized and incubated at 420C for 16 hours. Post hybridization wash was done in prewarmed wash solution 1 (0.4XSSC, 0.3% NP-40) at 73 0C for 2 minutes. Slides were then transferred to wash solution 2 (2X SSC,

0.1% NP-40) maintained at ambient temperature for 1 minute. After drying slides were counterstained with 4',6-diamidino-2-phenylindole (DAPI) at concentration of 125 ng/ml. 25 metaphases and 200 interphases were captured under epifluorescent microscope with the help of Isis—Metasystems software (Carl Zeiss Microscopy Gmbh, Göttingen, Germany).

Genomic DNA Extraction form peripheral blood

Genomic DNA was isolated from peripheral blood by salting out method (Miller et al., 1988). About 2.5 ml heparinized blood was mixed with 10 ml of 0.9% NaCl (4 times of blood). This mixture was incubated for 5 minute at room temperature and centrifuged at 5000 rpm for 5 minutes. The supernatant was discarded without disturbing the pellet. The pellet was broken down properly and mixed with 10 ml of solution A (4 times of blood). Mixing was done by gentle shaking for 5 minutes followed by centrifugation at 5000 rpm for 5 minutes. The supernatant was discarded, pellet was broken down again and 1 ml of solution B was added in it. Mixing was done for 5 minutes. 0.250 ml of solution C was added and mixed well. After mixing, 1 ml of chilled chloroform was added and mixed for 5 minutes. Centrifugation was done at 5000 rpm for 5 minutes. Aqueous layer was separated into another sterilized tube and equal volume of isopropanol was added to precipitate the DNA. DNA was transferred in to microcentrifuge tube and washed 2-3 times with 70% alcohol by centrifuging it at 2000-3000rpm for 5 minutes. The pellet was dried out at 37ºC for 2-3 hours. Obtained DNA pellet was dissolved in about 150 µl of TE.

Cytogenetic Microarray

Cytogenetic microarray experiment was performed using cytogenetic 2.7M array and Affymetrix microarray work station (Affymetrix, Inc. Santa Clara, CA,USA). The Cytogenetics 2.7M array provided whole genome coverage with 2.7 million markers to enable superior resolution. This array is also include 400,000 single nucleotide polymorphisms (SNPs) to enable the detection of loss of heterozygosity (LOH), uniparental disomy (UPD), and regions identical-by-descent. The Cytogenetics 2.7M array was used to detect known and novel chromosomal aberrations across the entire genome.

Whole genome amplification was performed using 99 ng of genomic DNA with the help of reagents provided in Cytogenetic 2.7M array kit (Affymetrix, Inc. Santa Clara, CA, USA). Amplified genomic DNA was purified by using cyto magnetic beads, cyto wash buffer and cyto elution buffer as per Affymetrix Cytogenetics Assay Protocol User Manual. Eluted DNA was checked for its optimum quality and quantity by OD260/280 ratio between 1.8-2.0 and DNA concentrations >0.55 µg/µL. Approximately 2 µg of purified DNA was fragmented and lebelled using cyto fragmentation and labeling buffers and enzymes. Fragmentation reaction was checked by running on 3-4% agarose gel electrophoresis. DNA fragments ranging from 50bp to 150 bp was considered as optimum fragmentation before proceeding for hybridization. For hybridization, fragmented samples were mixed with cyto hybridization buffer and denatured in thermal cycler. After denaturation, samples were immediately loaded in arrays and checked for any bubble (if bubble is there it should be movable). Arrays were placed in trays and rotated at 60 rpm in hybridization oven at 50 °C for 16 to 19 hours. After completion of hybridization arrays were subjected for washing and staining using GeneChip Fluidics Station 450. The standard bleaching protocol was run on the GeneChip Fluidics Station before using Fluidics Station for processing Cytogenetics Arrays. Bleaching, washing and staining were performed by the Affymetrix GeneChip Command Console (AGCC) software on GeneChip Fluidics Station 450. Washing and staining of hybridized arrays was done by using cyto stain buffer 1, cyto stain buffer 2, cyto holding buffer, cyto wash buffer A and cyto wash buffer B. After completion of washing and staining arrays were scanned by GeneChip Scanner 3000 7G with the help of software AGCC scan control. Cell files generated after scanning were run and analyzed in Chromosomal Analytical Suit (ChAS) software. For genomic duplication and deletion analysis filter was set at 400 kbp deletion and duplication standard.

RESULTS

Case 1 was referred with complaint of short stature at the age of 14 years girl for chromosomal diagnosis. Karyotyping revealed mosaicism for ring X chromosome with two types of cell line 45,X and 46,Xr(X) in the case (**Figure 1**). The mosaicism of the cell line with 46,Xr(X) in case 1, (**Table 1**). Origin of ring chromosome was determined by FISH using probe for centromeric region of chromosome X (**Figure 2**). Degree of mosaicism was further confirmed by scoring FISH signals on metaphases and interphases.

Cytogenetic microarray analysis revealed exact break points on both arms of X chromosomes in each case. In case 1, (**Figure 3**) the smallest r(X) chromosome (40Mb) with Xp deletion (Xpter-Xp11.3) of 43.58 Mb harbouring 375 genes and 79 Mb deletion of Xq (Xq21.1-Xq28) harbouring 1006 genes were observed (**Table 2**). Degree of mosaicism was also evident from cytogenetic microarray experiment on comparing deviation of probe signals from the base line in the case (**Figure 4**). Combining karyotype, FISH and cytogenetic microarray results case 1, was cytogenetically characterized as 45,X[120]/46,Xr(X)(p11.23q13.3)[78].

DISCUSSION

In present study the case of ring X chromosome with two types of cells cytogenetically characterized as 45,X and 45Xr(X) and the proportionate impact of genetic material loss was also reflected in the patient.

But it is difficult to ascertain the impact of mosaicism on clinical phenotype as level of mosaicism. Although, largest deletion in our cases was observed in case 1 (Xpter-Xp11.3 [43.58 Mb] and Xq21.1-Xq28 [79Mb]) but impact of this deletion on secondary sexual character could not be correlated in the patient because of younger age (14 years) at the time of referral. We correlated the only phenotype present in this case i.e. growth retardation with haplo-insufficiency of SHOX gene in pseudoautosomal region of Xp which has been identified to be associated with short stature phenotype (Rao et al., 1997; Clements Jones et al., 2000). Further, we compared Xp deletions and clinical manifestation observed in our cases with previous reported cases where there is no evidence for ovarian dysgenesis with an Xp terminal deletion (Pfeiffer, 1980; Wyss et al., 1982). Fertility has been reported to be maintained even when more than two third of the Xp is deleted (Lachlan et al., 2006). However, critical region for menarche proximal to Xp11.2 (Ogata et al., 2001) and a case having interstitial deletion between Xp11.2 and Xp11.4 with secondary amenorrhea (Zinn, 2001) have been reported. In our study, case 1 was presented with most proximal Xp deletion (Xp ter-Xp11.3) (Figure 4.3). Therefore, we considered ovarian dysgenesis in our cases is because of Xq deletion rather Xp deletion.

Before implication of fine molecular cytogenetic techniques several studies had reported karyotype-phenotype correlation for mosaic r(X) chromosome and suggested patients having small ring chromosome show relatively severe clinical features. It has been shown that the severity in cases with small ring X chromosome is because of failure of small ring chromosome to be inactivated due deletion of XIST at Xq13 (Matsuo et al., 2000; Tomkins et al., 2002). In our case XIST was intact with the ring chromosome in case negating role of XIST inactivation in clinical phenotype in patients (Figure 4.3).

Previous reports have suggested different critical region on Xq arm associated with development premature ovarian follicles (POF) which involve Xq13.3-q22. Alterations in these pathways are reported to be responsible for ovarian dysfunction.

Moreover, it has been observed that irrespective size of genetic content loss, majority of patient with Xq deletion have oligoamenorrhea followed by secondary amenorrhea or premature menopause (Therman et al., 1990). On comparing our cases with already reported cases of partial Xq deletion with ovarian dysfunction, it was observed that the same deletion can have different clinical consequences on ovarian dysgenesis similar to the observation reported by Maraschio et al., 1996. Therefore, ovarian dysgenesis related to chromosomal abnormalities can be due to disrupted expression of critical genes (Sala et al.,1997), inappropriate gene expression following incomplete paring of X chromosome at pachytene leading to meiotic arrest (Schlessinger et al., 2002) or position effect of the breakpoints on the flanking X-linked gene or autosomal linked genes.

It was concluded that ring (X) chromosomes lead to phenotypic heterogeneity which varies remarkably with its gene content and level of mosaicism. It becomes very crucial to determine exact rearrangement in the chromosome and level of mosaicism in cases with r(X) chromosome for genotype–phenotype correlation and do genetic counselling.

Conflict of Interest:

No

Authors' contributions:

PC: Data analysis, manuscript revision and corrections. **AKR:** Fund arrangement, major inputs, data interpretation, correspondence and corrections in the manuscript.

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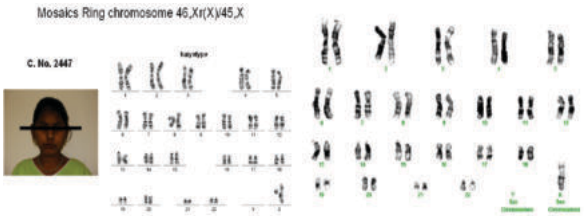

Figure 1: Clinical photo of girl and Karyotyping revealed mosaicism for ring X chromosome with two types of cell line 46,Xr/X 45,X and in the case 1

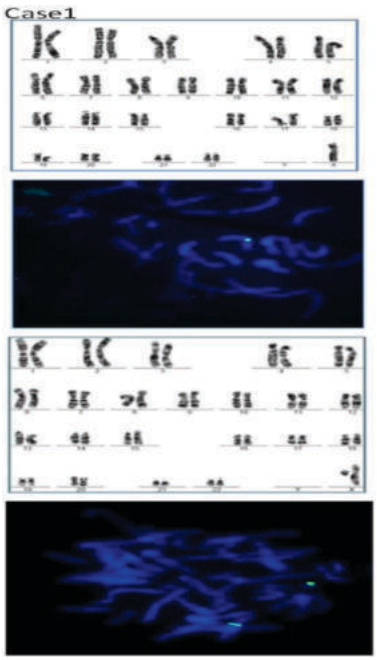

Figure 2: Panel with representative karyotype and FISH of case 1 showing presence of two cell lines and ring X chromosome.

Row A shows karyotype of the cases with 45, X cell line. Row B represents FISH on 45, X cell line hybridized with spectrum green labelled probe for pericentromeric region of chromosome X. Row C shows karyotype of another cell line with ring X chromosome r (X) present in all cases. Row D represents FISH on 46, Xr (X) cell line hybridized with the same probe showing two signals, one on chromosome X and another on r (X) chromosome.

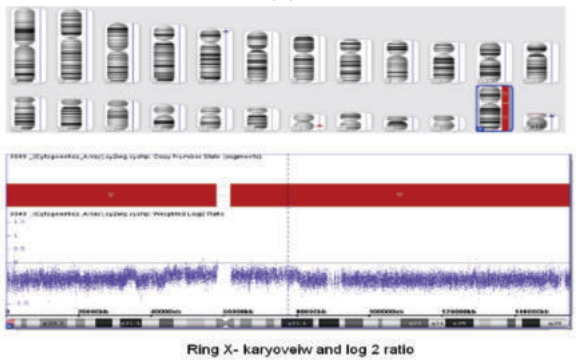

Figure 3: Cytogenetic microarray result representing log 2 ratio for deletion and breakpoints on Xp and Xq in the case 1 (blue colour probe signals). Representation of deletion and breakpoints in the case 1 (upper smooth signal and lower linear view).

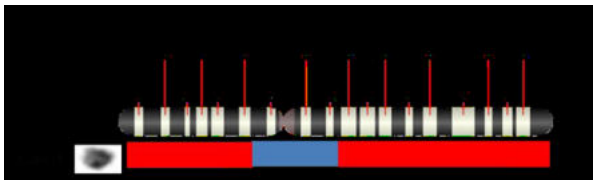

Figure 4: Sketch diagram showing breakpoints and deleted Xp and Xq arms in the case 1.

Ring chromosomes present in the cases are shown on the extreme left, red-coloured bar represents deleted terminal ends of X chromosome and blue-coloured bar represents intact portion with the ring chromosome.

Table 1: Clinical characteristics of girl as case 1

Case	1
Age (years)	14
Height (cm)	125
Weight (kg)	20
BMI (kg/m2)	12.8
Sexual development	Under developed

Table 2. Cytogenetic description of the case by microarray of case 1

Case ID	Cell types	Mosaicism for 46,X	Xp deletion	No. of Genes deleted on Xp	Xq deletion	No. of Genes deleted on Xp
1	45, X/46, Xr (X)	40%	Xpter-Xp11.3 (43.58Mb)	375	Xq21.1-Xq28 (79Mb)	1006

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