STUDY OF CHROMOSOMAL ABNORMALITIES IN RECURRENT PREGNANCY LOSS

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Abstract:
Cytogenetic and molecular cytogenetic characteristics have been studied in 200 patients with fertility problems. Chromosome analysis has been performed by GTG banding on the cultures of peripheral blood lymphocyte The purpose of the present study was to investigate the contribution of chromosomal anomalies and the frequency of a particular type of aberration in couples with recurrent miscarriages. First trimester pregnancy loss is a very common complication and a matter of concern for couples planning pregnancy. Balanced chromosomal rearrangements in either parent is an important cause of recurrent pregnancy loss particularly in the first trimester of pregnancy. Chromosomal analysis is an important etiological investigation in couples with repeated spontaneous abortions as it helps in genetic counseling and deciding about further reproductive options. We believe that the patients with chromosomal anomalies in the karyotype need differentiated treatment.

Keywords: Inversion, Fluorescent *in situ* hybridization, Polymerase chain reaction, Translocation, Karyotype, Lymphocyte culture.

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INTRODUCTION

A recurrent pregnancy loss or spontaneous abortion is the natural death of an embryo or fetus in the womb. Occurrence of miscarriage ranges up to 25% of all known pregnancies. The recurrent pregnancy loss is defined as three or more consecutive pregnancy loss that affects the couples trying to establish a family. Both genetic and non-genetic factors are responsible for recurrent pregnancy loss. The likelihood that a miscarriage is due to chromosome abnormality is a function of gestational age, with earlier gestations being more likely to be affected[1]. Couples with recurrent miscarriage are facing an increased risk of being carriers of a structural balanced chromosome abnormality. Balanced translocations reported 0.2% in neonatal population, 0.6% of infertile couples and upto 9.2% of patients with recurrent abortions[2]. A parental carrier of a structural chromosome rearrangement is associated with a history of recurrent pregnancy loss in 3.5 % couples[3].According to Dutta et al.[4] contribution of chromosomal abnormalities is as high as 70% in all pregnancies which end up as spontaneous miscarriages. Parental chromosomal abnormalities represent an important etiology of recurrent miscarriage; which is defined as a condition of three or more consecutive pregnancy losses before 24 weeks of gestation. The presence of chromosomal rearrangements can lead to unequal crossing over during meiosis which can result in gametes with unbalanced chromosomes like duplications or deletions. The clinical consequencesof such imbalances usually are lethal to developing embryoand leading to spontaneous miscarriage. Couples who have two or more miscarriages are at increased risk of carrying a structural chromosome abnormality in one of the partners. The incidence of carrier status of chromosome structural abnormality areincreases from approximately 0.7% in general population up to 2.2% after one miscarriage, 4.8% after two miscarriages, and 5.2% after three miscarriages[5].

Chromosomal abnormalities are the major contributors to the genetic cause of reproductive disorders and recurrent pregnancy loss. Therefore, it is important to analyze the incidence of genetic abnormalities and embryonic development in patients with recurrent pregnancy loss. Among the genetic factors, parental chromosomal abnormalities like insertion, deletion, inversion, translocation is one of the possible causes for recurrence pregnancy loss in the first three months of pregnancy.

The cytogenetic studies performed over the last decade in the couples with various reproductive dys-functions showed that each couple needs such diagnostics[2]. The patients with reproductive problems include patients with primary sterility, habitual pregnancy loss, and with a child born with many congenital malformations (MCM) and/or micro-anomalies of development (MAD) during anamnesis. According to the results of examinationsof couples with reproductive problems, the frequency of chromosomal anomalies varies from 4.3 to 9.6%[2&6]. It is produced due to a fact that sometimes during anaphasestage of cell division, a centromere abnormally splits transverse instead of longitudinal resulting in the formation of two daughter chromosomes of unequal lengths, each with duplication of genes[4].

MATERIALS AND METHOD

Total 200 patients (100 couples)who have two or more spontaneous abortionwere enrolled from Rama Medical College, Hospital&Research Centre and nearby adjoining areas. Chromosome analysis has been performed by GTG banding on the cultures of peripheral blood lymphocyte.Polymerase chain Reaction (PCR) with specific molecular markers for Y chromosome were also done when required to find the specific chromosomal abnormality and specific microdeletionsin Y chromosome on the same set of samplesand when structural chromosomal abnormality was recorded by the karyotype.

For karyotyping, venous blood of 2-3 mL was collected from both male and female partners in a sodium heparin vacutainer tube. 0.5 mL of whole blood was added in 5 mL of culture media (RPMI-1640) along with newborn calf serum 10% and 0.1 mL of phyto- hemagglutinin in a 15 mL conical centrifuge tube which was kept for 69 to 72 hours incubation at 37°C temperature in CO_2 incubator with 85% humidity and 5% concentration of CO_2 in slanting position. After 70th hour, the test tube was incubated for 1 hour after adding 100 µL of colchicine (0.1µgm/mL) and then centrifugation was done at 1000 rpm for 10 minutes in a centrifuge machine. The supernatant was then discarded by pipetting and added 7 mL of potassium chloride solution (0.56%) and the sample was incubated at 37ºC for 25 minutes. After 25 minutes, the cells were prefixed by using 3 mL Carnoy fixative solution (3Methanol: 1Acetic Acid) followed short incubation at 4ºC for 10 minutes to fix the cells. After 10 minutes centrifugation was repeated 3-4 times with steps until the cell pellet become

white. Later on, fixative solution (5 mL) was added finally to make harvested cells ready for slide preparation by drop method. Staining of the slide was done by Giemsa stain after treatment with trypsin known as GTG banding, trypsin digests the protein euchromatin and heterochromatin and formed the light and dark bands by using Giemsa stain.

Slides were observed under a bright field microscope attached to a camera and computer. Metaphase were photographed and a karyogram was prepared with the help of cytovision software. A total of 20 metaphase in the slide of each case were observed which were extended to 50metaphases in case of suspected mosaicism. Karyotypes were reported as per International System for Human Cytogenetic Nomenclature (ISCN, 2016) guidelines.

Y chromosome microdeletion was performed to analyze whether recurrent pregnancy loss is associated with deletions. The fluorescent signal intensity was detected in 4 channels Joe/Hex/yellow, Fam/Green, Rox/Orange, Cy5/Red.The result was interpreted using the real time polymerase chain reaction (PCR) instrument software comparing the crossing or not crossing of the threshold line by the fluorescence curve and the analysis of three samplesin which any deletion in chromosome Y. Chromosomal analysis is an important investigation with recurrent pregnancy loss.

RESULTS AND DISCUSSION

In the present study, total 200 samples (100 couples) who have two or more spontaneous abortion were enrolled for the study.All the cases were analyzed by karyotype and three cases were analyzed by Real Time Polymerase Chain Reaction (RT-PCR) method which were morphologically found abnormal by karyotype.The cases which were identified as abnormal by karyotype are listed in Table 1for age, gender, indications, result of karyotype and its interpretation. Total 34 cases were identified abnormal from karyotype between the age of 24 to 45 years. All 34 cases were reported for recurrent pregnancy loss or repeated spontaneous abortion. The recurrent pregnancy loss was reported mainly at advanced maternal age.

Table 1.Identified 34 abnormal cases out of 200 Samples

S.No.	Age in years/Gender	Indications	Karyotype	Interpretation
1	32 Y/F	Recurrent pregnancy loss	Abnormal	Female 46,XX, add(17)(p13.1)
2	29 Y/F	Repeated spontaneous abortion	Abnormal	Female 46, XX,inv(9)(p11q12)
3	27 Y/F	Recurrent pregnancy loss	Abnormal	Female 45,XX,t(13;14)(q10;q10)
4	31 Y/F	Recurrent pregnancy loss	Abnormal	Female 46,XX,15ps+,22ps+
5	29 Y/F	Repeated spontaneous abortion	Abnormal	Female 45,XX,rob(14;22)(q10;q10)
6	38Y/F	History of abortions	Abnormal	Female46,XX,t(2;3)(p25;p21)
7	26Y/F	History of abortionsConsanguineous marriage	Abnormal	Female46,XX,1qh+
8	29Y/F	History of abortions	Abnormal	Female46,XX,inv(9)(p11q12)
9	27Y/M	History of abortions	Abnormal	Male47,XY,+mar
10	26Y/F	Recurrent Pregnancy Loss	Abnormal	FemaleTranslocation 13;14 45,XX,t(13;14)(q10;q10)
11	31Y/F	Recurrent pregnancy Loss	Abnormal	Female47,XXX
12	32Y/F	History of abortions	Abnormal	Female46,XX,22ps+
13	31Y/F	History of abortions	Abnormal	Female45,XX,rob(14;21)(q10;q10)
14	28Y/M	History of abortions	Abnormal	Male46,XY,14ps+
15	37Y/M	History of abortions	Abnormal	Male46,XY,inv(9)(p11q12)
16	33Y/F	Repeated spontaneous abortion	Abnormal	Female46,XX,14ps+
17	35Y/M	Recurrent pregnancy Loss	Abnormal	Male46, XY,t(2;22)(p21;p13)
18	24Y/F	Repeated spontaneous abortion	Abnormal	Female46, XX,inv(9)(p11q12)
19	30Y/F	Recurrent pregnancy Loss	Abnormal	Female46, XX, 22ps+

20	32Y/M	History of abortions	Abnormal	MaleVariant inversion Y 46,XY,inv(Y)(p11.2q11.23)
21	28Y/M	History of abortions	Abnormal	Male47,XY,+mar
22	26Y/F	History of abortions	Abnormal	Female46,XX,22ps+
23	32Y/M	Repeated spontaneous abortion	Abnormal	Male46,XY,15ps+
24	35Y/F	Recurrent pregnancy, Advance maternal age	Abnormal	Female46,XX,t(3;10)(p10;p10)
25	33Y/M	Recurrent pregnancy Loss	Abnormal	MaleVariant inversion Y 46,XY,inv(Y)(p11.2q11.23)
26	40Y/M	Repeated spontaneous abortion	Abnormal	Male46,XY,t(7;10)(p21.3;q21.1)
27	40Y/M	History of abortions	Abnormal	Male46,XY,15ps+
28	32Y/M	Recurrent pregnancy Loss	Abnormal	Male46,XY,t(4;5)(q22;p15.3)
29	28Y/F	Repeated spontaneous abortion	Abnormal	Female46, XX,t(4;13)(p15.2;q22)
30	25Y/F	Recurrent pregnancy Loss	Abnormal	Female46, XX, t(4;18)(p15.2;p11.3)
31	39Y/F	Repeated spontaneous abortion, Advance maternal age	Abnormal	Female46, XX,t(10;11)(q11.2;q25)
32	32Y/M	Repeated spontaneous abortion	Abnormal	Male45, XY, t(13;14)(q10;q10)
33	31Y/M	Recurrent pregnancy Loss	Abnormal	MaleVariant inversion Y 46,XY,inv(Y)(p11.2q11.23)
34	45Y/M	Repeated spontaneous abortion and Advance Maternal Age	Abnormal	Male46, XY,t (5;12)(q22 ; q13)

Comparison of ChromosomalAbnormality

In karyotype study, total thirty-four cases are found abnormal out of 200 cases and by the study of Real time PCR for Y chromosome, there is no any deletion in the chromosome Y; only inversion in chromosome Y was reported by karyotyping. In the present study, 2.94% abnormalities inaddition of genetic material; trisomy at 47,XXX. Total 5.88% abnormalities in marker chromosome; 8.83%abnormalities in chromosome inversion Y; 11.76% abnormalities in chromosome inversion 9; 14.71% abnormalities in Robertsonian translocation, 23.53%, Chromosome with satellite and maximum 26.47 for Balanced translocation (Table 2).

Table 2. Abnormality types recorded in 34 cases out of 200 samples

S. No.	Types of Abnormalities	Karyotype	Percentage
1	Addition	1	2.94
2	Trisomy 47, XXX	1	2.94
3	Marker Chromosome	2	5.88
4	Chromosome Inversion 9	4	11.76
5	Chromosome Inversion Y	3	8.83
6	Balanced translocation	9	26.47
7	Robertsonian translocation	5	14.71
8	Chromosome with satellite	8	23.53
9	Chromosome with polymorphism	1	2.94
Total		34	100

Chromosomal Abnormalities

The incidence of cytogenetic abnormalities in couplesby with age is given in table 3.Out of 200 cases (100 couples) studied, 14 females and 3 males were from the age group of ≤25 years, 47 females and 30 males were in the range of 26-30 years, 27 females and 38 males were falling in 31-34 years age group, 12 females and 23 males were from age group 35-40 years and 6 males were of the age >40 years. Among all these five age groups, chromosomal abnormality was seen as 10% &15.78% in 26-30 years and 31-34 years respectively in male partners, whereas 14.28%, 19.14% &22.22% were seen in ≤25 years, 26-30 years and 31-34 years respectively in female partners within the age group. The chromosomal abnormality was observed 17.39% in male and 25.00% in female partners in the age group of 35-40 years. It was also seen that; chromosomal anomalies were 16.66% present in age <40years in malesonly while no abnormality was observed in female age above 40 years (Table 3; Figure 1). The age above 35 years is advanced maternal/paternal age where the quality ovum becomes meagre due to abnormal gametogenesis. Previous studies on couples with defective reproductive success reported prevalence ranging from 2.4 - 13.1% in which one of the partners was the carrier for a balanced chromosomal rearrangement in contrast to an incidence of less than 0.55% in the general population[7-12].Indeed, since the development of cytogenetic analysis in 1970s, banding patterns has been the primary tool for the clinical assessment of patients with a variety of congenital anomalies[13]. G-banding of chromosomes promises to be the most valuable

technique for routine chromosome analysis due to its inherent simplicity, sensitivity, and stability of the material obtained. The results obtained by cytogenetic technique suggest that banding represents a native conformational feature of chromosomes[14]. Therefore, it was decided to study chromosomal analysis from peripheral blood lymphocytes with repeated pregnancy loss, according to standard cytogenetic methods using G-banding technique.

Table 3. Chromosomal Anomalies in males and females (34 Cases): Correlation with Age

Age (years)	Male (n=100)		Female (n=100)	
	Total	Abnormality [no. (%)]	Total	Abnormality [no. (%)]
≤25	3	0(0.0)	14	2(14.28)
26-30	30	3(10.0)	47	9(19.14)
31-34	38	6(15.78)	27	6(22.22)
35-40	23	4(17.39)	12	3(25.00)
>40	6	1(16.66)	0	0(0.0)

Figures in parentheses are percentages (%)

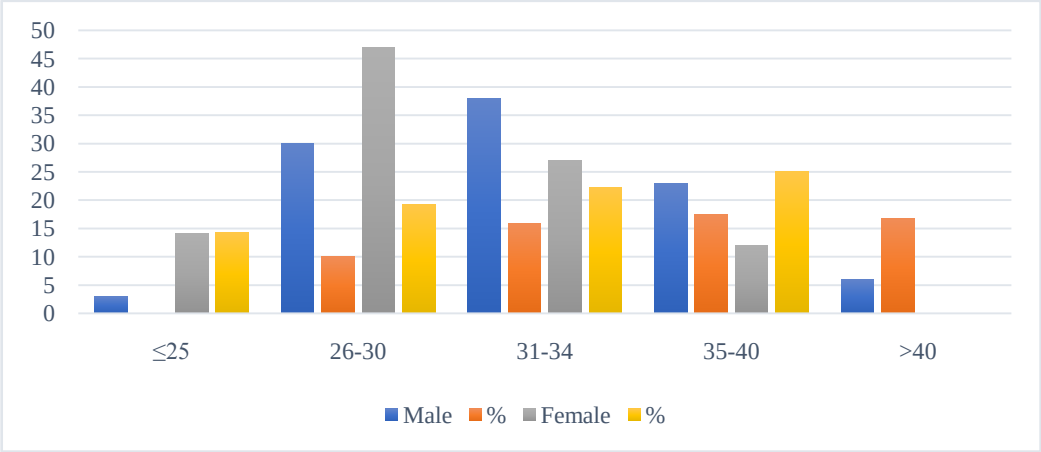

Figure 1. Chromosomal anomalies in male &females

Chromosomal Abnormalities with respect to Advanced Maternal Age

Total 8 cases (4%) were reported out of 200 abnormal in advanced maternal/paternal age depicted in table 4. The chromosomal abnormality was observed 17.39% in male and 25.00% in female partners in the age group of 35-40 years. It was also seen that; chromosomal anomalies were 16.66% present in age <40 years in males only while no abnormality was observed in female age above 40 years (Table 3). The possible reason may be due to imbalance segregation of gametes during meiosis with the formation of abnormal gametes in gametogenesis. The quality of gametes in advanced maternal/paternal age become miserable.

Table 4. Abnormalities with advanced maternal/paternal age

S.No.	Age/Gender	Observation	Interpretation
1	38Y/F	Balanced translocation 2 & 3	46,XX,t(2;3)(p25;p21)
2	37Y/M	Abnormal	Inversion 9 46,XY,inv(9)(p11q12)
3	35Y/M	Balanced translocation 2 & 22	46,XY,t(2;22)(p21;p13)
4	35Y/F	Balanced translocation 3 &10	46,XX,t(3;10)(p10;p10)
5	40Y/M	Balanced translocation 7 &10	46,XY,t(7;10)(p21.3;q21.1)
6	40Y/M	Abnormal	46,XY,15ps+
7	39Y/F	Balanced translocation 10&11	46,XX,t(10;11)(q11.2;q25)
8	45Y/M	Balanced translocation 5 &12	46,XY,t (5;12)(q22 ; q13)

Chromosomal Translocation

In the present study, 14 cases were reported for balanced translocation age group range from 25 to 45 years (5 males & 9 females) (Table 2). The type of translocation in 14 cases are listed in table 5 with age, gender, observation and its interpretation. The incidence of chromosomal abnormality was testified higher in female among the couples with recurrent pregnancy loss. The reason of this mechanism is the production of single ovum each month. However, millions of sperms release in every expulsion, so the nature select against the abnormal gametes.

Table 5. Type of Chromosomal Translocation

S.No.	Age/Gender	Observation	Interpretation
1	27Y/F	Robertsonian translocation 13 & 14	46,XX,t(13;14)(q10;q10)
2	29Y/F	Balanced translocation 14 & 22	45,XX,rob(14;22)(q10;q10)
3	38Y/F	Balanced translocation 2 & 3	46,XX,t(2;3)(p25;p21) RPL
4	26Y/F	Balanced translocation 13&14	45,XX,t(13;14)(q10;q10)
5	31Y/F	Balanced translocation 13&14	45,XX,rob(14;21)(q10;q10)
6	35Y/M	Balanced translocation 2 & 22	46, XY,t(2;22)(p21;p13)
7	35Y/F	Balanced translocation 3 &10	46,XX,t(3;10)(p10;p10)
8	40Y/M	Balanced translocation 7 &10	46,XY,t(7;10)(p21.3;q21.1)
9	32Y/M	Balanced translocation 4 &13	46,XY,t(4;5)(q22;p15.3)
10	28Y/F	Balanced translocation 4 &13	46, XX,t(4;13)(p15.2;q22)
11	25Y/F	Balanced translocation 4 & 18	46,XX,t(4;18)(p15.2;p11.3)
12	39Y/F	Balanced translocation 10&11	46, XX,t(10;11)(q11.2;q25)
13	32Y/M	Balanced translocation 13&14	45,XY,t(13;14)(q10;q10)
14	45Y/M	Balanced translocation 5 &12	46,XY,t(5;12)(q22 ; q13)

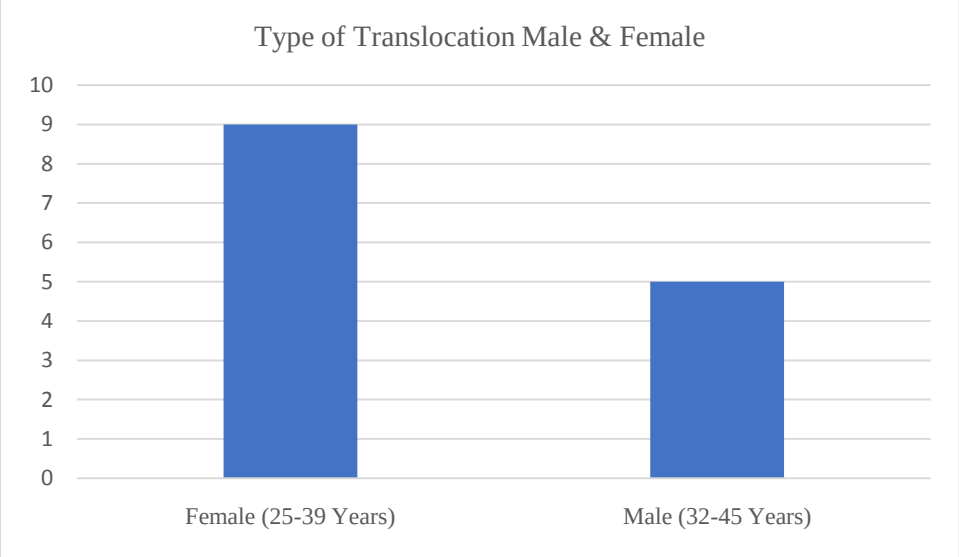

Figure 2. Translocations in Male & Female in correlation with age

The present study recognized the relation of chromosomal abnormality with advanced maternal age (Table 4). But Kochhar *et al.*[15] found that the risk of having a chromosomal aberration was not related to the number of previous miscarriages. It may be due to difference in case selection criteria in their study, because bias in patient selection can also eliminate some couples with a higher risk of a chromosome abnormality. Goncalves *et al.*[16] found an association between chromosomal abnormalities and in cases of recurrent miscarriage in first trimester of pregnancy similar to current findings. Boue et al.[17] quoted that, the incidence of chromosomal abnormalities detected after only two losses was 8-15 times higher than that of the general population. Percentage of abnormal karyogram in present study was 5.1% and 7.4% in cases with previous 2 and 3 or more abortions respectively, when low percentage mosaicism was considered normal. According to Tharapel et al[13] it was controversial whether to consider apparent low grade mosaicism for 45,X as abnormal case. The present study showed that the percent chromosomalaberrations were 8.5% with previous 2 first trimester abortions, 5.3% with 3 previous abortion history, 50% in couples with history of 4 first trimester abortions and 25% with previous ≥5 first trimester pregnancy losses, indicating that finding of chromosomal aberration chances mayincrease with history of multiple pregnancy losses[18-19].

Recurrent pregnancy loss can occur due to wide variety of reasons and is a challenging reproductive problem for the couples as well as the clinician. Approximately 40%-50% etiology of RPL is remaining unclear. Consanguineous marriage plays a role in chromosomal aberration, although it does not significantly seem among the population.Advanced maternal age and obesity is another factor which significantly associated with RPL. The most frequent deletion type is the AZFc region deletion (approximately 80%) followed by AZFa (0.5-4%), AZFb (1-5%) and AZFbc (1-3%). Deletions which are detected as AZFabc are most likely related to abnormal karyotype such as 46, XX male or iso(Y).

In the present study, total 7 abnormal inversion cases were reported in the range of 24 to37 years. The inversion cases have dominancy of inversion 9, 5 out of 7 were reported for inversion 9 (Table 6). The eight cases were reported for abnormal

with satellite in the range of 28-40 years. Total 3 males and 5 females were reported for satellite as 15ps+,22ps+; 46,XX,22ps+; 46, XY,14ps+ and 15ps+ (Table 7). Total three cases were reported for abnormality in chromosome Y (male) with variant inversion Y in the age group 31-33 years (Table 8).

Table 6. Abnormal Inversion Cases

S.No.	Age/Sex	Observation	Interpretation
1	24Y/F	Abnormal	Inversion 9
2	29Y/F	Abnormal	Inversion 9
3	29Y/F	Abnormal	Inversion 9
4	31Y/M	Inversion Y	Variant inversion Y
5	32Y/M	Inversion Y	Variant inversion Y
6	33Y/M	Inversion Y	Variant inversion Y
7	37Y/M	Abnormal	Inversion 9

Table 7. Abnormal Cases with Satellite

S.No.	Age/Sex	Observation	Interpretation
1	26Y/F	Abnormal	46, XX,22ps+
2	28Y/M	Abnormal	46, XY,14ps+
3	30Y/F	Abnormal	46, XX,22ps+
4	31Y/F	Abnormal	46,XX,15ps+,22ps+
5	32Y/M	Abnormal	46,XY,15ps+
6	32Y/F	Abnormal	46,XX,22ps+
7	33Y/F	Abnormal	46,XX,14ps+
8	40Y/M	Abnormal	46,XY,15ps+

Table 8. Abnormality in Chromosome Y

S.No.	Age/Sex	Observation	Interpretation
1	31Y/M	Inversion Y	Variant inversion Y
2	32Y/M	Inversion Y	Variant inversion Y
3	33Y/M	Inversion Y	Variant inversion Y

A statistically significant correlation was found between the number of previous abortions and the occurrence of chromosomal abnormalities in the study of Al-Hussain *et al.*[20]. While study conducted by El-Dahtory[21] in Egyptian couples concluded that the chromosome abnormalities were 7.4% with a history of two abortions, 13% with three abortions and in 17.39% with four or more abortions. Finding of increased incidence of chromosomal abnormality with increase in the number of previous first trimester abortions indicated that, this factor was significant to see abnormal cases.All the variable percentin the present study may be due to the fact that different populations vary in the incidence of carriers of chromosomal aberrations[20]. The female to male ratio 2.1:1[22] goes in parallel with current findings, suggesting distribution of chromosomal anomalies in males and females near to the ratio of 1:2.

CONCLUSION

Results of the present study show that the abnormalities vary between parents at different maternal and paternal ageand prevalence of chromosomal abnormalities found in consistent with other populations studies around the world. The balanced translocations are commonly observed in advanced maternal/paternal age. The overall incidence of chromosomal abnormalities indicates that chromosomal analysis of the couples with recurrent miscarriage should be essentially considered. Further precise molecular characterization of these chromosomal breakpoint regions could pave way for identification of new genes or genes involved in recurrent miscarriage and also help in elucidating the molecular mechanism underlying the aberrations. Hence testing of parents and the fetus demonstrates to be important for future pregnancies. Several genes have been studied in association with advanced maternal/paternal age and found the various reasons of abnormality. Findings of the present study may be useful to obstetricians and gynecologistand also to physicians in predicting future pregnancy loss and outcome of the progeny, which may acquire unbalanced chromosomal abnormalities from their parents, who have chromosomal aberrations in balanced form or in the form of mosaicism.

Conflict of Interest: The authors declare that there is no conflict of interest.

Declaration: The study has been conducted at the Dept. of Biochemistry, with collaboration of Dept. of Obstetrics & Gynecology of Rama

University, Kanpur, Uttar Pradesh India on the basis of detailed history, including their family history and thorough examination. A prior approval was taken by the Ethical Committee of Institution. A written informed consent, in the vernacular language, was obtained from all the participants, upon fulfilling the inclusion criteria.

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