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DETECTION AND ANALYSIS OF CHROMOSOMAL ABNORMALITIES IN PATIENTS WITH RECURRENT PREGNANCY LOSS USING CYTOGENETIC TECHNIQUES

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ABSTRACT

Recurrent pregnancy loss is known as post-implantation failures in natural conception which is identified as three or more consecutive miscarriages in a woman. Chromosome abnormalities, untreated hypothyroidism, uncontrolled diabetes mellitus, a few uterine structural anomalies, and antiphospholipid antibody syndrome are identified as the leading causes of recurrent pregnancy loss. 50%-60% of early spontaneous miscarriages are associated with chromosomal anomalies. This report highlights the detection of chromosomal abnormalities in couples with recurrent miscarriages using cytogenetic analysis. Cytogenetic analysis of miscarriages can explain at least 50% of cases. This project was carried out with a clinically recognized cohort of 10 couples that were referred to the Human Genetic Unit in Colombo. Karyotyping was performed for each partner in these couples, and analysis was done using GenASI 7.2.7 software. With the observed results, this report presents various types of chromosomal abnormalities that could lead to recurrent miscarriages. These patients could be educated and advised on possible treatments and genetic counselling to reduce the risks of the condition and assists in successful pregnancy outcomes.

Keywords: Cytogenetics, Karyotyping, Miscarriage, Chromosomal abnormalities

INTRODUCTION

The term "recurrent miscarriages" refers to pregnancies that end three times or more before 28 weeks from the last menstrual period (El Hachem et al., 2017). The risk of subsequent miscarriages increases with maternal age and previous miscarriages (Andersen et al., 2000). The increasing age-related risk of miscarriage for women who are over 35 years old is even more serious, according to the Royal College of Obstetricians and Gynaecologists (RCOG) guideline data, given that the likelihood of conception decreases with age in this age range (Turesheva et al., 2023). This project proposes to carry out a cytogenetic study on recurrent miscarriages to detect chromosomal abnormalities in couples who experience recurrent miscarriages. The identification of these chromosomal anomalies aids in determining the root cause and in providing patients with prognostic information (Hyde and Schust, 2015). Genetic counsellors can communicate with the affected couple about their risks of passing on the detected abnormalities, their chances of losing any future pregnancies, and the potential for giving birth to a live-affected kid (Kohn et al., 2016).

LITERATURE REVIEW

The most frequent causes of recurrent miscarriages are parental chromosomal abnormalities, uncontrolled diabetes,

untreated hypothyroidism, certain uterine structural defects, and antiphospholipid antibody syndrome (APS) (Larsen et al., 2013). In addition to that, endocrine disorders, thrombophilias, immunologic abnormalities, and environmental causes are other possible reasons (Ford and Schust, 2009). However, about 40%–60% of recurrent miscarriage cases are idiopathic (Hodes-Wertz et al., 2012).

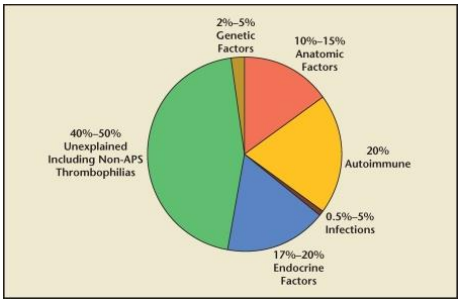


Figure 1: Several potential etiological reasons for RPL, include genetic, anatomical, autoimmune, infectious, endocrine, and even unidentified and unexplained causes (Ford and Schust, 2009).

Parental chromosomal abnormalities are seen in 2%-8% of couples who experience recurrent miscarriages (Ocak, Özlü and Ozyurt, 2013). Chromosomal abnormalities can be both structural and numerical. The most prevalent type of structural chromosomal aberration is known as translocation that include both balanced and unbalanced translocations (Aygun, 2017).

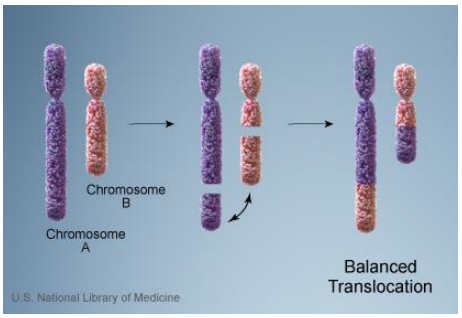


Figure 2: Chromosome segment dissociates and attaches to another chromosome in a process known as balanced translocation (Salem, 2016)

Most of the chromosomal abnormalities are balanced chromosomal translocations (Ocak, Özlü and Ozyurt, 2013). A balanced translocation can be detected in 5% of couples who suffer recurring losses (Priya et al., 2018). In addition, among couples who have repeated abortions, 40% of translocations are Robertsonian and 60% are reciprocal translocations (Wan et al., 2021). A balanced translocation is nearly two times more likely to occur in females than in males (Kavalier, 2005). When chromosome sections move around on the chromosomal map in a balanced manner, no significant genetic material is lost or gained. One in 500 people has a balanced translocation (Stephenson and Sierra, 2006). Unbalanced gametes are produced as a result of balanced chromosomal defects, and this might lead to repeated miscarriages. It results in infertility, frequent abortions, or the birth of infants with birth defects (Kochhar and Ghosh, 2013).

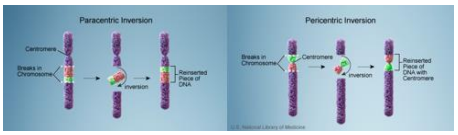


Figure 3: Inversion is when a section of DNA on a chromosome is oriented differently from a reference karyotype or genome (Salem, 2016).

Inversions are another chromosomal abnormality linked to recurrences of pregnancy loss. When there are two breakpoints in the chromosome, that particular region rejoins the chromosome after a 180-degree rotation and creates a mutation in the chromosome. It doesn't show any abnormalities at the phenotypic level (Li et al., 2023). Heterochromatic

expansions also contribute to the occurrence of spontaneous abortions. Repetitions of Specific DNA regions may have an effect during meiosis, which may decrease reproductive capacity (Dong et al., 2013). A study carried out by (Lei, Zhang and Zheng, 2022) to differentiate the proportion of chromosomal abnormalities observed in products of conception (POCs) among sporadic and recurrent pregnancy loss. This study confirms that the chromosomal abnormalities were much more frequent in spontaneous pregnancy loss than they were in recurrent pregnancy loss. The prevalence of chromosomal abnormalities was found in POCs reduced with an increase in the number of pregnancies lost and was lower in recurrent pregnancy loss than in random pregnancy loss (Lei, Zhang and Zheng, 2022).

In (Pal et al., 2018) a study on chromosomal abnormalities showed afflict between 2% and 8% of couples who experience recurrent pregnancy loss, making it a difficult reproductive issue. Recurrent miscarriages (RM) are often caused by chromosomal abnormalities, notably balanced translocation rearrangements in either parent. 17 couples out of 172 showed various chromosomal structural or numerical abnormalities. Eight of the seventeen couples had balanced translocations, two had Robertsonian translocations, five had pericentric inversions of chromosomes 8, 9, and Y, and only two women had abnormalities in the number of the sex chromosomes(Pal et al., 2018). Another study carried out by (Kocaaga, Kilic and Gulec, 2022) about the chromosomal abnormalities pattern in RM. There are numerous recognized non-genetic aetiologies for RPL at present. These include uncontrolled diabetes, untreated hypothyroidism, specific structural abnormalities of the uterus, and antiphospholipid antibody disorder. 2.2% of the structural chromosomal abnormalities were found in this study. In fact, Robertsonian translocations were found in 1.4% of the couples in this study, then reciprocal translocations in 0.8% of the couples (Kocaaga, Kilic and Gulec, 2022).

METHODOLOGY

Specimen collection

This retrospective study was carried out at the Human Genetics Unit, Department of Anatomy, Faculty of Medicine, University of Colombo. Informed, written consent was obtained from all patients before the collection of samples.

Culturing

Culturing peripheral blood lymphocytes was done using peripheral blood from samples from patients referred to the Human Genetics Unit. Fresh venous blood was collected in a heparin tube for karyotyping. Blood culturing was performed under laminar air flow in sterilized conditions. To prevent contamination, the workstation was cleaned with 70% ethanol. Two samples were needed from each patient. First, 250 µl of peripheral blood was added to a sodium heparin tube, which contains 5 ml of PB Max karyotyping media. Then it was kept in a CO2 incubator for 72 hours. Two separate cultures were carried out.

Harvesting

At the end of the 71st hour, 50 µl of Ethidium Bromide was added to the samples. Those were incubated in CO2 for 30 minutes. Then 50 µl Colcemid was added to the incubated samples. Again, samples were incubated in CO2 for 30 minutes and 72 hours. Samples were centrifuged for 10 minutes at 1200 rpm. The resulting pellet was treated with 5 ml of KCl while being vortexed after the supernatant was removed. The samples were kept in a water bath at 37°C for 30 minutes. Then 500µl of the chilled fixative agent, which was prepared using

methanol: acetic acid, 3:1, was added to the sample while vortexing. Centrifugation was performed at 1200 rpm for 10 minutes. After removing the supernatant, the pellet was transferred into a 1.5-ml microcentrifuge tube. A fixative agent was added, and it was kept for 10 minutes at room temperature. This was centrifuged at 6000 rpm for 2 minutes. Then to the pellet, 1000µl of fixative agent was added, and it was kept for 10 minutes at room temperature. A clear solution was obtained after repeating these last two steps a few times. Then it was stored at 4°C for 1 hour. The sample was then centrifuged at 6000 rpm for 2 minutes. 1 ml of fixative was added to the sample and then stored at -20ºC until use.

Slide preparation

Initially, the water bath temperature was adjusted to 92°C–94°C, and the humidity in the room was adjusted to 79°C–82°C. Then the pipette tips were cut off slightly. The sample was mixed well before use. The rough side of the slides was held face down on the steam of a hot water bath for 3 seconds. Then slides were placed on the holder in such a manner that the rough side faced up. After that, 35 µl of the sample was added to both edges of the slide. Then 35µl of the fixative agent was added on top of the samples as one drop for each edge. Slides were covered with a lid for 1 minute. Then slides were kept on the dryer for 1 hour until they stained.

Slide staining

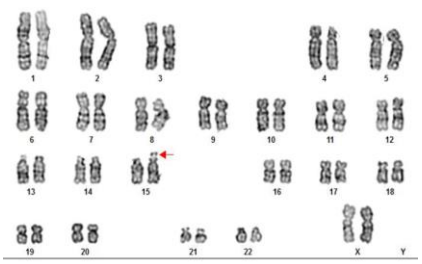
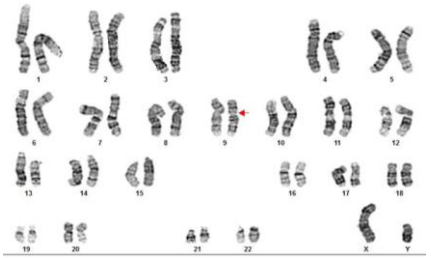
The standard Giemsa Trypsin Leishman (GTL) banding staining technique, which is followed by the Human Genetics Unit, University of Colombo, was used. Gurr buffer was prepared by dissolving 1 tablet in 1000 ml of distilled water, and PBS buffer was prepared by dissolving 1 PBS tablet in 500 ml of distilled water. Using an electronic balance, 0.019g of Tripsin was measured, and it was dissolved in 60 ml of PBS buffer. Then 3 ml of Gurr buffer was mixed with 1 ml of Leishman stain. The two slides were placed on the rack horizontally. The full amount of Tripsin was splashed onto the slides, and at the same time, the stopwatch was switched on. At 6 seconds, slides were washed by dipping them in distilled water once. Then the prepared stain was added to the slides, and the slides were kept for 1 minute. Each slide was rinsed twice in Gurr buffer. After that, the slides were placed in the oven for 15 to 30 minutes.

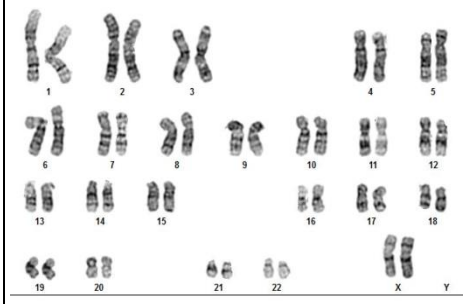
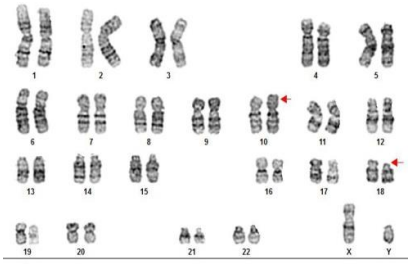
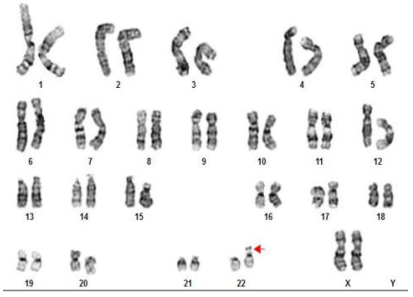
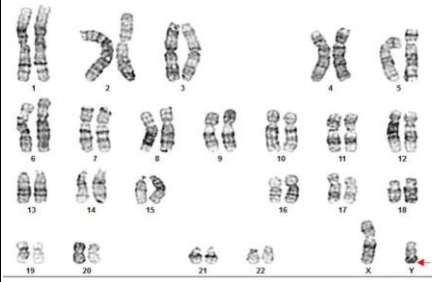
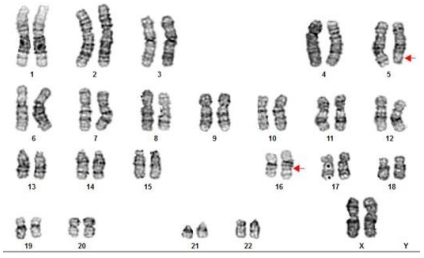
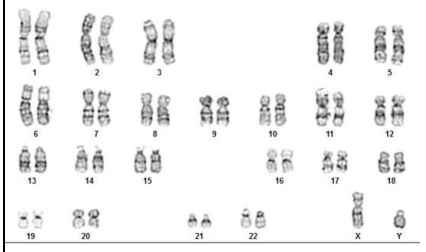
Chromosome analysis

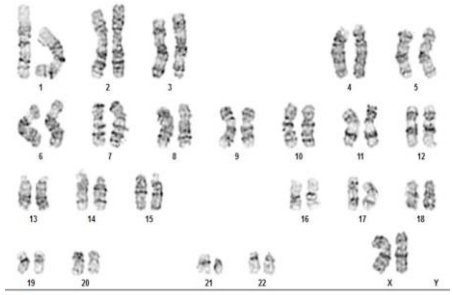
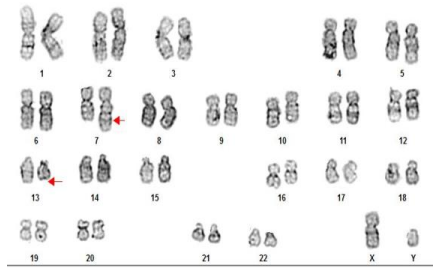
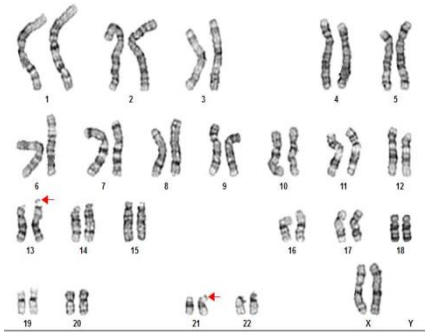
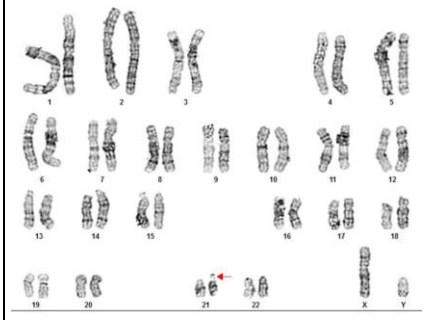
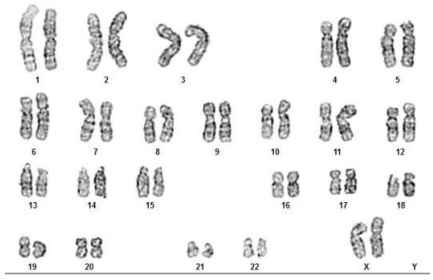
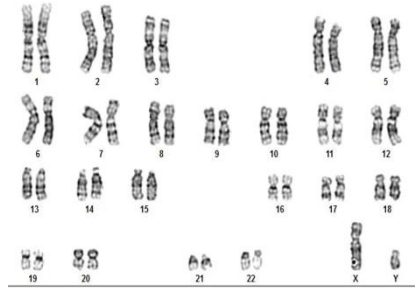
About 25-40 metaphases were captured using the Olympus BX61 microscope. These were karyotyped and analyzed using the GenASIs 7.2.7 software.

DATA ANALYSIS

Table 1: Results obtained from the karyotype analysis of 10 couples recognized with RPL

Couple A	46,XX,15pstk+ 	46,XY,inv(9)(p13;q13) 

Couple B	46,XX 	46,XY,t(10;18)(p13;p11.2) 
Couple C	46, XX,22pstk+ 	46, XY,Yqh+ 
Couple D	46,XX,t(5;16)(q33;q24) 	46,XY 

Couple E	46,XX 	46,XY,t(7;13)(q32;q14) 
Couple F	46,XX,21pstk+ 	46,XY,21pstk+ 
Couple G	46,XX 	46,XY 

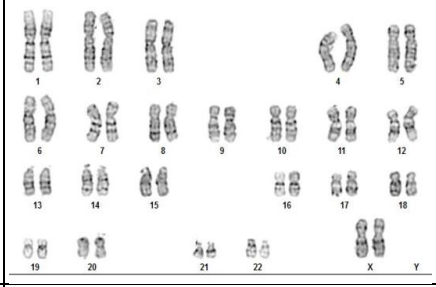
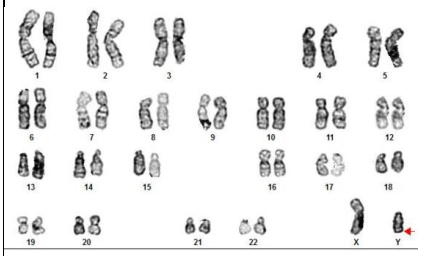
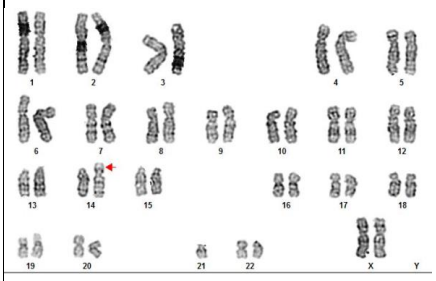
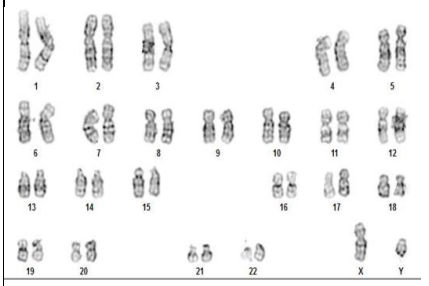
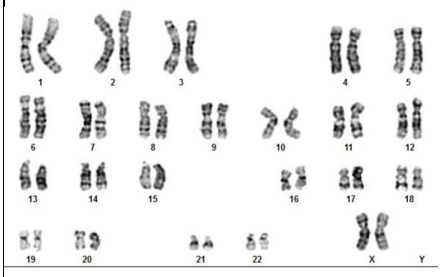
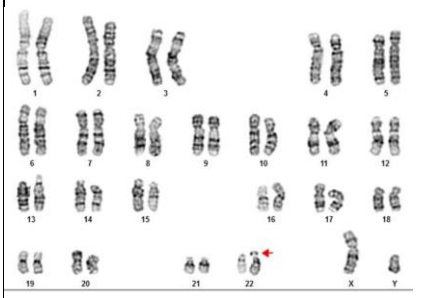
Couple H	46,XX 	46,XY,Yqh+ 
Couple I	45,XX,rob(14;21)(q10;q10) 	46,XY 
Couple J	46, XX 	46,XY,22pstk+ 

Table 2: Summary of the chromosomal abnormalities observed

Couple	Female	Male
A	Presence of stalk 15pstk+	presence of 9 chromosome inversion inv(9) (p13:q13)
B	Normal	Presence of translocation t(10;18)(p13;p11.2)
C	Presence of stalk 22pstk+	Heterochromatic expansion Yqh+
D	Presence of translocation t(5;16)(q33;q24)	Normal
E	Normal	Presence of translocation t(7;13)(q32;q14)
F	Presence of stalk 21pstk+	Presence of stalk 21pstk+
G	Normal	Normal
H	Normal	Heterochromatic expansion Yqh+
I	Presence of translocation rob(14;21)(q10;q10)	Normal
J	Normal	Presence of stalk 22pstk+

DISCUSSION

Cytogenetic techniques were applied to study chromosomal abnormalities in a clinically defined cohort of couples with recurrent pregnancy losses. Ten couples were screened for karyotyping analysis. In all the couples, at least one of the partners had some abnormality in their karyotype. These abnormalities produce imbalanced gametes, which can lead to reproductive issues such as repeated miscarriages and infertility, even though most carriers do not experience any phenotypic effects (Mogib El-Dahtory, 2011). As summarized in Table 2, Reciprocal translocations, Robertsonian translocations, inversions, and stalks were the chromosomal abnormalities that were detected in this cohort which were identified as structural abnormalities. According to the results observed, male

partners in couples B and E and female partners in couples D and I were presented with translocations. Three of them were reciprocal translocations, which were observed among chromosomes 10, 18, 5, 16, 7, and 13. Robertsonian translocations were observed in female partner of couple I, on chromosomes 14 and 21.

Translocation takes place when a portion of one chromosome is transferred to another chromosome. Recurrent miscarriages occur more frequently and infants with abnormal genetic makeup are more likely to be born in couples with balanced reciprocal translocations (De, Chakravarty and Chakravarty, 2015). Chromosome breakpoints are the basis for the formation of balanced, unbalanced, and normal gametes (Priya et al., 2018). Repeated abortions due to reproductive errors are occurring in response to sequence rearrangements of respective functional genes (Priya et al., 2018). There is evidence based on present studies that meiotic errors result in the generation of unbalanced gametes (Ananthapur et al., 2012) Chromosomes 13, 14, 15, 21, and 22 are mostly involved in Robertsonian translocation (Miryounesi et al., 2016). The involvement of 13q, 14q, and 21q in the translocation corresponds to 75% and 13%, respectively (Choi et al., 2013). In couple A, male partner possessed a pericentric inversion of chromosome 9 in his karyotype. An inversion is a type of rearrangement where a chromosomal region is turned around completely. There won't be any genetic information lost, but the linear sequence might vary as a result (Kirkpatrick, 2010). Inversion can be pericentric or paracentric. Inversions compromise double breakpoints, and after breakage, the segment is reinserted in a 180° orientation. If the centromere is included in the segment, it is known as pericentric, and if not, it is known as paracentric inversion (Gonçalves et al., 2014). The deletion or suppression of euchromatin chromosomal regions during this process could result in defects in the embryo (Sheth et al., 2013). It is thought that 1-3% of the general population has pericentric inversions on chromosome 9, which is highly structurally polymorphic and most frequently affected (Xie et al., 2020). As long as rearrangement is balanced, inversion usually does not cause any abnormality. According to recent research, heterozygous people produce more aberrant chromatids, which lowers fertility by causing the development of imbalanced gametes (Dana, Stoian and Mierla, 2012).

A Y-chromosome polymorphism was another anomaly found. It describes the chromosome's expansion, contraction, or repetition (Wang et al., 2017). Male partners of couples C and H were found to have expanded Y chromosomes. It is reported that the incidence of occurring heterochromatic variations in the Y chromosome is responsible for spermatogenesis (Posam and Sabnis, 2016). Therefore, abnormalities in the structure of the Y chromosome are related to infertility and recurrent miscarriages. Studies have demonstrated that miscarriages originate from mitotic mistakes caused by an increase (Yqh+) or decrease (Yqh-) on the long arm of the Y chromosome (Dong et al., 2013). Increased heterochromatin length in these two karyotypes may result in chromosome non-disjunction, embryonic chromosomal disorders, and ultimately miscarriages (Wang et al., 2017). Stalks and satellites are another heterochromatic variation that was observed in couples who experienced recurrent miscarriages. Within this cohort, the stalk on the p arm of chromosome 15 was observed in the female partner of couple A. The female partner of couple C and the male partner of couple J possessed stalks on the p arm of chromosome 22. Karyotypes of both partners in couple F presented stalks on the p arm of chromosome 21. Satellites and stalks are highly repeated sequences of DNA, and

they do not encode proteins (Hong et al., 2011). These polymorphic variations are considered normal, and there is no phenotypic effect (Mierla and Stoian, 2012). But recent studies have suggested that these variations give rise to different abnormal reproductive outcomes, such as infertility and recurrent pregnancy miscarriages (Hong et al., 2011). Couple G presented with no single chromosome abnormality; thus, they experienced recurrent miscarriages. This could indicate the association of the recurrent pregnancy loss experienced with other etiological factors that are not genetic. It can be anatomical, endocrine, infectious, immunological, thrombotic, environmental, or have other unexplained etiologies (Ford and Schust, 2009).

Genetic counseling is particularly crucial to inform the patient about the clinical characteristics of the condition, recurrence risks, and potential treatments once the couple is diagnosed as having the disease and the cause is determined to be genetic abnormalities. That could assist the patient with available options for reproduction. Other than that, prenatal genetic studies such as chronic villus sampling and amniocentesis can be used during pregnancy to check the genetic makeup of the offspring (Wieacker and Steinhard, 2010). Additionally, in vitro fertilization with preimplantation genetic testing is possible. For translocation carriers who experience multiple miscarriages, it is suggested as a quicker way to conceive a live child than natural conception (Keymolen et al., 2012). In this process, women take series of medications for several days and it results in the growth of many eggs in ovaries. These eggs are retrieved from the ovaries and one sperm is injected into each egg and is allowed to grow into an embryo. One cell from the embryo is used to analyze the genetic makeup of the embryo before transfer to the womb using preimplantation genetic screening (Munné, 2002). The idea of preimplantation genetic screening has been established with new technologies including trophectoderm-laser-assisted blastocyst biopsy and molecular karyotyping using whole genome amplification and either comparative genomic hybridization (CGH) or single nucleotide polymorphism (SNP) arrays (Keymolen et al., 2012). Therefore, genetic evaluation and counselling of couples with recurrent miscarriages is more important for better reproductive outcomes.

CONCLUSION

According to the results observed within the cohort, there were translocations, inversions, stalks, satellites, and heterochromatic expansions. The female partner in couple D and male partners in couples B and E were presented with reciprocal translocation, while the female partner in couple I was presented with Robertsonian translocation. A pericentric inversion was found on chromosome 9 in the male partner of couple A. Stalks were observed on chromosome 15 in the female partner of couple A and on the 22nd chromosome in the female partner of couple C and the male partner of couple J. Stalks were found in both partners in couple F on the 21st chromosome. Heterochromatic expansion in the q arm of the Y chromosome was found in male partners in couples C and H. All these patients were clinically recognized for recurrent miscarriages.

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