RESEARCH ARTICLE

Cytogenetic analysis in couples with recurrent miscarriages: a retrospective study from Punjab, north India

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Abstract

Human reproduction is considered as the most inefficient event as ~15–20% of human pregnancies end in miscarriage and in the product of miscarriages, chromosomal anomalies are a common occurrence. The aim of the present retrospective study was to assess the frequency of chromosomal aberrations in couples with recurrent miscarriages in the region of Punjab and to compare with worldwide frequencies. In this study, a total of 440 cases were referred between the period 1995–2015. After lymphocyte culturing, giemsa–trypsin banding was done for each case to assess the chromosomal anomalies. The frequency of chromosomal aberrations among couples was found to be 3.41% in our study. Among these aberrations, balanced reciprocal translocations formed the largest group with 60% anomalies. We would conclude that clinicians should understand the importance of chromosomal analysis in these couples and refer them for karyotyping after two miscarriages to rule out the possible genetic cause of recurrent miscarriages.

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Introduction

The most common outcome of conception is embryonic or foetal demise viewed as nature's quality control for selecting genetically normal offspring. Recurrent miscarriage (RM) is defined as the loss of three or more consecutive pregnancies before 20 weeks of gestation and about 60% of RM might be caused by chromosomal aberrations in the embryo (Carp *et al.* 2004). Determining the exact cause of RM is difficult to ascertain due to its multifactorial nature; such as advanced maternal and paternal age, endocrine dysfunction, autoimmunity, infectious diseases, environment toxins, congenital, uterine anomalies and genetic abnormalities (Dudley and Branch 1989).

In couples experiencing miscarriages, the percentage of chromosomal rearrangements has been found to be 5.5% as compared to 0.55% of the general population (Fryns *et al.* 1984) and in the product of conception (POC) of miscarriage, chromosomal abnormality is found to be 50–70%. It is documented that this chromosomal abnormality may be due to a balanced reciprocal translocation carrier parent or might result from a recurrent numerical abnormality, which is usually not inherited, but may cause recurrent miscarriage (Nussbaum *et al.* 2004; Driscoll and Gross 2009). Moreover, the carriers, having chromosomal rearrangements are at a higher risk of producing unbalanced gametes which can lead to sterility, RM and giving birth to malformed children. The evident chromosomal abnormalities and balanced chromosomal rearrangements observed in couples with recurrent pregnancy loss (RPL) are considered to be reliable aetiologies, but its implementation in preimplantation genetic diagnosis remains controversial, as stated by Ozawa *et al.* (2008) and Sugiura-Ogasawara *et al.* (2004). On contrary, Fischer *et al.* (2010) proposed that PGD (prenatal genetic diagnosis) would benefit the pregnant carrier couples with history of RPL and significantly improving the rate of successful pregnancies. Even though several structural rearrangements occur *de novo*, the larger part appears to be familial, further, it is important to do cytogenetic analysis of the couple to rule out the possibility of structural rearrangements and genetic counselling is connoted for couples who have experienced more than two pregnancy losses.

RPL can be physically and emotionally rendering for couples and may feel the guilt of being incomplete as they cannot create a viable pregnancy (Ford and Schust 2009). Routine cytogenetic analysis of miscarriages remains an uncommon

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practice till today. This unfortunate omission has impacted the management of couples with RM (Yassen *et al.* 2001; Stephenson *et al.* 2002). The aim of the present retrospective study was to evaluate the frequency of chromosomal anomalies in couples subjected to RPL in Punjab, India. This study may facilitate the clinicians in the region by improving their knowledge in context of chromosome abnormalities with respect to cases with repeated miscarriages and can also generate baseline data regarding choromosomal aberrations in context of RM in Punjab.

Materials and methods

A total of 440 couples (880 individuals) from different districts of Punjab having at least two consecutive miscarriages were referred for cytogenetic analysis from 1995 to 2015. After taking the written informed consent; clinical and medical history was noted on a predesigned proforma along with three generation pedigree to assess any history of disease and consanguinity. Peripheral blood lymphocyte cultures based on phytohaemiagglutinin stimulation were set up after procuring 3 mL of peripheral heparinized blood (Moorhead *et al*. 1960 (with modifications)). G-banded karyotyping was done using trypsin–giemsa banding preparations. At least, 25 metaphases were scanned for each individual with Olympus BX51 microscope and metaphases were karyotyped using Cytovision software. These anomalies were reported according to International System for Human Cytogenetic Nomenclature (ISCN 2013) (Shaffer *et al.* 2013). The study was approved by ethical committee of the Guru Nanak Dev University.

Results

In this study, the mean age of women was found to be 27.9 years (21–44) and the mean age of men was 32.4 (20–46). The mean number of miscarriages in these women was found to be 4.3. Women above the age of 35 were found to be 10%. The mean duration of marriage was 5.2 years. In 10% of cases, a positive family history of RM was revealed through pedigree analysis. The mean age of females at menarche was 12.67 years, by recall method (ranging 10–16). All the females in the present study were nonsmokers and nonalcoholics, whereas the percentage of male smokers was 15% and that of alcoholics was around 30%. The percentage of males who were both alcoholics and smokers were 13%. In this study, toxoplasmosis, rubella, cytomegalovirus, herpes simplex (TORCH) infections, anatomical problems, endocrinological disorders were found to be in 47, 24 and 18%, respectively (the values are rounded off) (table 1).

Of the total 440 couples, the percentage of couples carrying chromosomal aberrations were found to be 3.41%. The frequency of chromosomal aberrations in all the individuals was 1.71%. The average number of miscarriages in the cases was found to be 3.3. The chromosomal anomalies are represented in table 2. The percentage of males (78%) carrying translocations was higher than females (22%). The male to female rearrangement ratio was found to be 1.5 : 1 in the present study. The balanced reciprocal translocations formed a bigger group (60%) in case of cytogenetic anomalies in the couples. In the present study, 26.6% cases showed involvement of chromosome 4 and 13.3% of the cases showed association of chromosome 6 in reciprocal balanced translocations. In the present study the frequency of satellite chromosomal associations (D–D; D–G; G–G) was found to be 26.36%. Of these acrocentric associations, D–G associations dominated the group with 68% followed by G–G and D–D with 16.4% and 15.5%, respectively. The following cases showed various cytogenetic anomalies.

Table 1. Different factors involved in pathogenesis of RM.

Factor	Percentage (round off)
TORCH	47
Uterine abnormalities	24
Endocrinological disorders (hypothyroid)	18

Table 2. Showing the chromosomal anomalies in couples with RM.

Chromosomal anomalie	Karyotype	Total number of cases
Structural anomalies		
Reciprocal translocation	46,XY,t(4;8)(p16;q13) 46,XX,t(11;21)(p15.5;p11.4) 46,XY,t(4;15)(q15.3;p13) 46,XY,t(5;9)(q22;q34.3) 46,XX,t(3;6)(q29;q14) 46,XY,t(6;11)(q14;p15) 46,XY,t(8;13)(q13;q34) 46,XY,t(19;22)(p13.3;p13)	8
Robertsonian translocation	46,XY,t(13;22)(p10;p10)	1
Duplications and inversion	46,XX,dup(4)(p14;p15.2) 46,XX,dup(22)(q12;q13)? 46,XY,inv(4)(p11;q22)	3
Polymorphic variants	46,XY,Yqh+ 46,XX, 15ps+	3
Satellite associations		116

A phenotypically and anatomically normal nonconsanguineous couple with a history of seven repeated consecutive miscarriages was referred for cytogenetic analysis. The female aged 30 reported dysmennorhea. The chromosomal constitution revealed a balanced translocation in husband: 46,XY,t(19;22)(p13.3;13). The male aged 33 reported alocohol consumption once a week.

Another phenotypically and anatomically normal nonconsanguineous couple presented with a history of three consecutive miscarriage in first trimester. The husband aged 30 reported alcohol consumption, but was nonsmoker. The female aged 33 was TORCH positive and had normal chromosomal constitution, whereas translocation was observed in spouse: 46,XY,t(4;8)(p16;q13) through cytogenetic analysis.

A husband (34) and wife (33) who are nonconsanguineous, phenotypically and anatomically normal reported with two consecutive miscarriages and one live birth. The

wife had no endocrinological dysfunction. Chromosome analysis revealed a mosaic pattern of karyotype in 64% metaphases of male i.e. 46,XY/46,XY,t(5;9)(q22;q34.3). The female had normal chromosomal constitution.

A translocation: 46,XX,t(3;6)(q29;q14) observed in a female aged 34 who was presented with three consecutive miscarriages in first trimester (figure 1). She has one child alive and is phenotypically normal. The nonalcoholic and nonsmoker male partner (aged 37) was phenotypically and chromosomally normal.

This is a case where a three generation translocation was observed in the family. A nonconsanguineous couple was referred for genetic counselling after three consecutive miscarriages. The product of conception analysis revealed a translocation between chromosomes 6 and 11 in the foetus. The mother (aged 26) also showed the mosaic constitution: 46,XX,t(6;11)(q14;p15). Cytogenetic investigations in grandparents (carried out by us) revealed that maternal grandfather was harbouring the same translocation (figure 2).

A nonconsanguineous, phenotypically and anatomically normal couple (husband 28 years and wife 26 years) with no family history of miscarriage was presented with three repeated spontaneous miscarriages. Cytogenetic findings revealed translocation: 46,XY,t(8;13)(q13;q34) in the male counterpart, whereas the female was having normal karyotype.

A balanced reciprocal translocation in male (40): 46,XY, t(4;15)(q15.3;p13) was seen in a nonconsanguineous couple with a history of 10 repeated miscarriages and one live birth. No chromosomal anomaly was seen in wife (36), but reported positive for TORCH infection. The male was alcoholic with frequency of alcohol consumption once a month.

A phenotypically normal, nonconsanguineous couple having three repeated miscarriages was referred for chromosomal analysis. The couple had no family history of miscarriage. The chromosomal analysis revealed a balanced reciprocal translocation in the female (aged 26): 46,XX,t(11;21)(p15; p11.4), and the ultrasound revealed a small fibroid, whereas the husband aged 28 had normal chromosomal constitution.

A Robertsonian translocation: 46,XY,t(13;22)(p10;p10) was seen in a male (27 years) in the cytogenetic findings of a nonconsanguineous, phenotypically and anatomically normal couple with a history of three repeated miscarriages in first trimester.

The couple married for three years presented with a history of two consecutive miscarriages in first trimester. The female aged 28, suffered from hypothyroidism was put on thyroid medication. Cytogenetic investigation of the couple revealed a normal chromosomal constitution in nonalcoholic and nonsmoker male. A polymorphic variant was observed in female i.e. 46,XX,15ps+ (figure 3a).

A duplication: 46,XX,dup(4)(p14;p15.2) was seen in wife, whereas no anomaly was seen in husband. The couple had a history of seven miscarriages. They were nonconsanguineous, phenotypically and anatomically normal.

Another couple presented with a history of four miscarriages was phenotypically and anatomically normal. In the cytogenetic analysis a duplication: 46,XX,dup(22)(q12;q13) was observed in the female aged 36, whereas the male was of normal chromosomal constitution i.e. 46,XY.

A polymorphic variant was observed in the husband's Y(Yqh) chromosome (figure 3b) through chromosomal analysis in two nonconsanguineous couples. The first couple presented with bad obstetric history, their first child died three

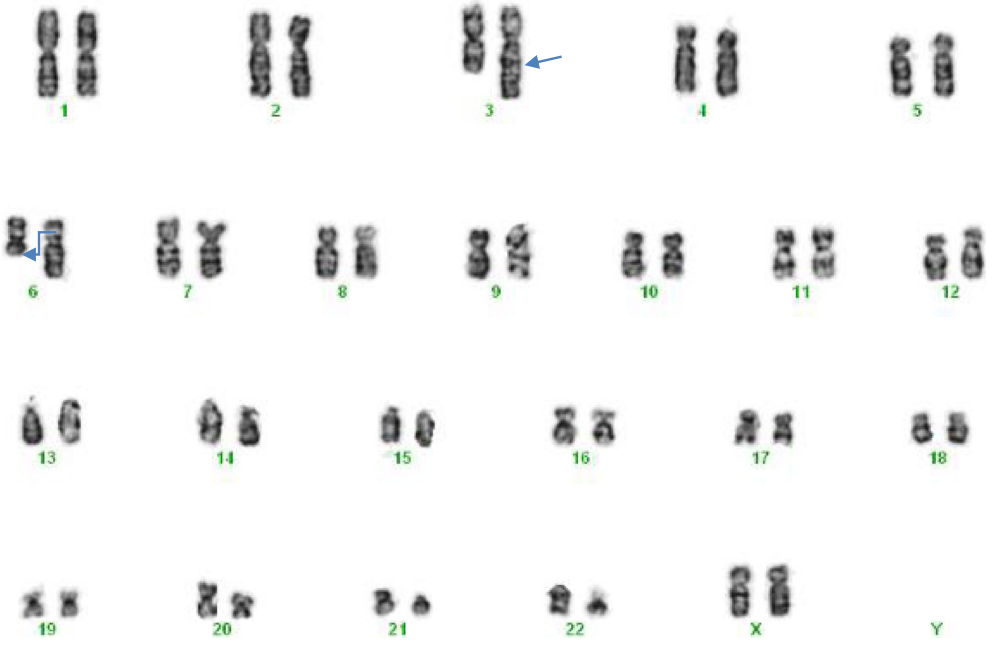

Figure 1. Showing translocation 46,XX,t(3;6)(q29;q14).

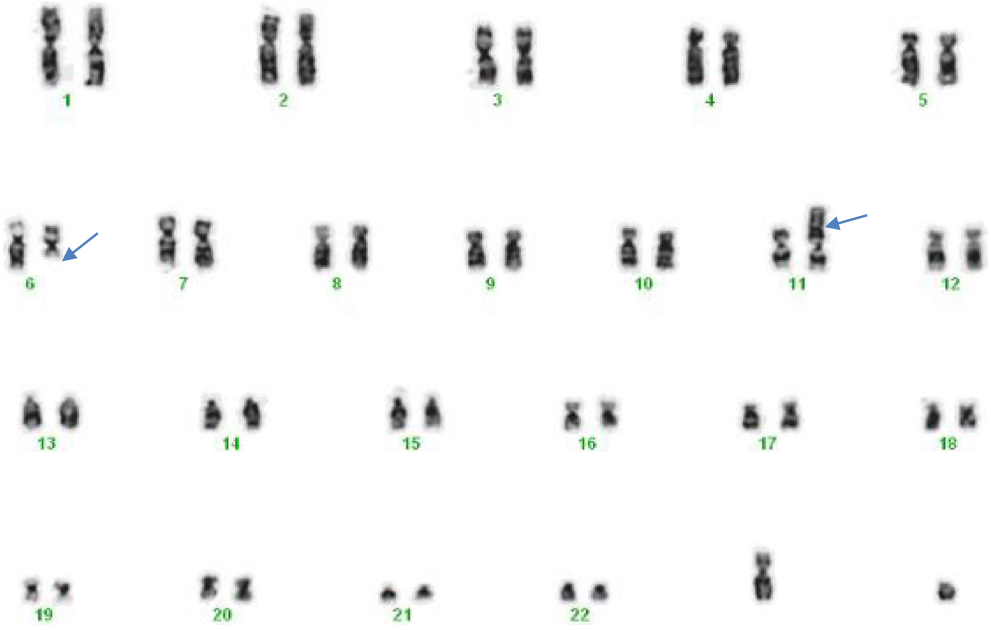

Figure 2. Showing translocation 46,XY,t(6;11)(q14;p15).

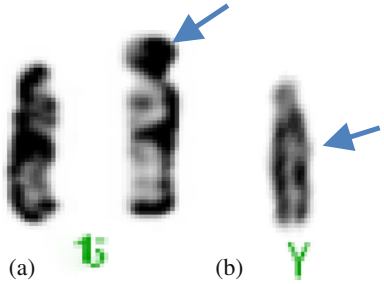

Figure 3. Heteromorphic polymorphism in (a) chromosome 15ps^{+} and (b) chromosome Yqh^{+}.

days after birth and two conceptions lead to spontaneous miscarriages in first trimester. The male reported consumption of alcohol on daily basis. The second couple had a history of three consecutive miscarriages. The females showed normal chromosomal constitution.

The couple's first conception occurred after four years of marriage but the couple was phenotypically and anatomically normal. They were referred for cytogenetic analysis after two miscarriages, which revealed an inversion in chromosome 4 in the male (aged 30) counterpart 46,XY,inv(4)(p11;q22).

Discussion

Human reproduction is characterized by its inefficiency as substantial percentage of all conceptions fails to reach a live birth. Approximately, 15–20% of all clinically recognized pregnancies end up with miscarriage, and the total pregnancy loss is estimated to be 30–50% (Rai and Regan 2006; Stephenson and Kutteh 2007). The reason for majority of miscarriages in the 10th week of gestation can be attributed to foetal aneuploidy, whose background lies in the errors during the first meiotic division of the oocyte, which is initiated in the womb of mother and is incomplete until ovulation (Jacobs and Hassold 1987). In balanced translocations, two chromosomes exchange their segments which lead to structural chromosomal rearrangements, relatively a frequent phenomenon.

Recurrent miscarriages are conventionally defined as three or more consecutive pregnancy losses prior to 20–22 weeks of gestation, but now-a-days, even two consecutive miscarriages are considered for further evaluations as the subsequent risk of pregnancy loss after two consecutive miscarriage rises to about 25% and after three miscarriages, it is 33% (Coulam 1991). RM is a multifactorial condition with several causes such as genetic makeup of parents, uterine abnormalities, hormonal imbalances, haematological disorders, immunological disorders and environmental factors adding to the aetiology. The spontaneous abortion in the first trimester is due to chromosomal anomalies, skewed X-chromosome inactivation, genomic imprinting, single gene mutation, chromosomal instability and sperm chromosomal abnormalities, which explain idiopathic reproductive loss (Dutta *et al.* 2011).

Our study is the first of this kind from this region. In our study, the mean maternal age of subjects carrying anomalies was 27.9 years. In this study, 10% females were above the age of 35. Advanced maternal age has been associated with increased number of miscarriages (Rocherbrochard and Thonneau 2002), but we could not find correlation of

maternal age with the number of abortions observed in these subjects indicating that the chromosomal abnormalities could arise because of some reasons other than maternal age. According to Hook and Cross (1983), the risk of chromosomal abnormalities is 1/476 at the age of 25, whereas the risk increases with advancing maternal age at delivery which is 1/385 at 30, 1/196 at 35, 1/66 at 40. In the present study, the incidence of chromosomal aberrations was found to be 3.41% in couples, while the frequency in other couples of different populations varies from 2–9.92% (table 3). One cumulative study done by Kalvana *et al.* (2004) reported an average of 2.8% of chromosomal aberrations in couples with recurrent abortions, which is not very different from the frequency in the present study. The frequency of chromosomal abberations in India as given by Dubey *et al.* (2005) was 2%. Highest frequency in couples with RM was observed by Tsui *et al.* (1996) in China which is found to be 9.92% followed by 9.04% by Nazmy (2008) in Egypt. The variable prevalence in several studies might be related to the different sample size, ethnicity, consanguinity, social and other criteria used for the investigation of the cases.

The male to female ratio of chromosomal rearrangement is 1.5 : 1 in our study not consistent with other studies, except from one by Testart *et al.* (1996), who reported high frequency of translocations and inversions (3.6 : 1) in males as compared to females, underwent intracytoplasmic sperm injection treatment. A lower fertility rate can be speculated with male reciprocal translocation carriers because of poor motility reported in sperms with high frequency of structural chromosomal abnormalities (Rybouchkin *et al.* 1997).

The type of abnormality plays an important role in the effect of aberration. Fryns *et al.* (1988) have reported that of all chromosomal abnormalities in couples with recurrent abortions, two-thirds were balanced autosomal translocations. It has been estimated that the risk of miscarriage in couples with reciprocal translocations is ∼25–50%, whereas it is ∼25% with Robertsonian translocation (Lee and Silver 2000). Balanced translocations account for the largest percentage of these karyotypic abnormalities. They can cause pregnancy loss, because segregation during meiosis results in gametes with duplication or deficiency of chromosome segments. In the present study, 60% reciprocal translocations formed the major group. Frequently involved chromosomes in the present study were 4, 15, 22, 6, 11 and Y, whereas out of the balanced reciprocal rearrangements, chromosome 4 was involved in 26.6% of cases. Chromosome 4 has been reported in bad obstetric history by Neu *et al.* (1979), Karakus *et al.* (2012), De *et al.* (2015) and Sheth *et al.* (2015). Translocation between chromosomes 6 and 11 has also been observed by Dutta *et al.* (2011), Karakus *et al.* (2012) and Ghazaey *et al.* (2015), in couples with recurrent miscarriages. We observed that acrocentric chromosomes were involved in 40% of total aberrant cases, of these chromosomes 15 and 22 were intricated in 50% of cases. The chromosomes 15 and 22 have been implicated in many studies with reproductive failure (Mozdarani *et al.* 2008; Dutta *et al.* 2011; El-Dahtory 2011; Ghazaey *et al.* 2015). The size of the chromosomal segment involved in the frequency of the breakpoints and their positions have a vital role in reproduction. In translocations, breakpoints are nonrandom, especially in couples with bad obstetric history (Campana *et al.* 1986). The absence of phenotypic manifestations in balanced reciprocal translocation carriers and its possible consequence of giving birth to children with unbalanced chromosomal rearrangements makes karyotyping essential diagnostic tool for couples with RM.

Table 3. Global frequencies of chromosomal anomalies in different populations.

Study	Country	Couples	Chromosomal abnormality (%)
Present study (2015)	India (Punjab)	440	3.41
Dubey *et al.* (2005)	India (New Delhi)	742	2
Sheth *et al.* (2013)	India (Gujarat)	4859 individuals	3.5
Dutta *et al.* (2011)	India (south)	1162	3.35
Al-Hussain *et al.* (2000)	Saudi Arabia	193	3.88
Azim *et al.* (2003)	Pakistan	300	2.65
Ghazaey *et al.* (2015)	Iran (northeastern)	728	5.85
Niroumanesh *et al.* (2011)	Iran (Tehran)	100	6
Nazmy (2008)	Egypt	376	9.04
Pal *et al.* (2009)	Malaysia	56	4.45
Tsui *et al.* (1996)	China	512	9.92
Makino *et al.* (1990)	Japan	639	5.0
Goud *et al.* (2009)	Sultanate of Oman	380	6.84
De Braekeleer and Dao (1990)	Canada	22199	4.7
Flynn *et al.* (2014)	UK	795	3.52
Fryns and van Buggenhout (1998)	Belgium	1743	5.34
Celep *et al.* (2006)	Turkey	645	3.86
Elghezall *et al.* (2007)	Tunisia	1400	6.93
Gadow *et al.* (1991)	Argentina	682	6.8

We observed polymorphic variants in three cases (20%) of which two were Y chromosome heterochromatin variants. The role of these polymorphic variants of chromosomes in reproductive failure has been supported by Boronova *et al.* (2015). These heterochromatic polymorphisms, large satellites and fragments have been implicated in mitotic instability and a tendency towards an increased risk for aneuploidy (Ward 2000).

Satellite associations (SA) were seen in 26.36% cases in our study. Hassold and Jacob (1984) reported that acrocentric chromosomes were involved in one-third of trisomies observed in spontaneous abortions and live borns. It has been proposed that the presence of nucleolar organizer regions (NORs) on the short arms of all five acrocentric chromosomes predispose them to nondisjunction (Schmickel *et al.* 1985; Garcia *et al.* 1989). The high satellite association tendency may influence the risk of nondisjunction was strongly supported by the fact that the SA tendency of chromosome 21 of the parents with nondisjunction was significantly increased when compared with a control group as studied by Hansson and Mikkelson (1974).

TORCH infections were reported in 47% of cases in our study. According to Charles and Larsen (1990), it is very unlikely that maternal infection causes recurrent abortion. Li *et al.* (2002) carried out tests for TORCH screen and did not find any positive result among 200 patients over a five-year period, and further suggested that infections do not play a significant role in RM. Infections have been considered as an occasional cause of sporadic miscarriage (Summers 1994; Li *et al.* 2002).

In the present study, in 24% of cases, uterine anomalies (septate uterus, uterine fibroids, ovarian cysts and endometriosis) were detected. An observational data by Homer *et al.* (2000) suggested that a septate uterus was associated with an increased risk of miscarriages. Better pregnancy outcomes have been reported after surgical interventions (Christiansen 2006). Age at menarche in the present study was found to be 12.67 years. According to Bracken *et al.* (1985) and Abetew *et al.* (2011), younger age of menarche i.e. less than 11 years is associated with pregnancy complications. So the age at menarche did not play a role in RM in the present study.

The percentage of endocrine dysfunctions was found to be 18% in our study. Endocrine disturbances have been implicated to cause recurrent miscarriage. A meta-analysis by Prummel and Wiersinga (2004) supported an association between thyroid autoantibodies and history of miscarriages.

Apart from routine analysis of the couples regarding anatomical, endocrine and infections, these data suggest that cytogenetic evaluation is necessary for an accurate approach to elucidate the cause of recurrent miscarriage. Genetic counselling with an option of prenatal genetic diagnosis should be offered to couples with chromosomal aberrations as it significantly reduces the losses and increases the rate of viable pregnancy (Otani *et al.* 2006).

Summary and conclusion

This study is first of its kind from the region of Punjab reporting chromosomal aberrations in recurrent miscarriage couples and has tried to create a baseline data which can assist the physicians by increasing their awareness about the nature and frequency of chromosomal aberrations. Karyotyping of parents of the affected person and product of conceptions could not be performed to find out paternal or maternal origin of the abnormality and then to possibly relate the presence of abnormality as a cause of miscarriage.

Repeated miscarriages in couples are enigma as often the aetiology remains unanticipated in most of the cases. We have observed in our study that mostly clinicians do not refer the cases for chromosomal analysis until the couples have undergone the trauma of third miscarriage. So, likely we conclude that clinicians should understand the importance of chromosomal analysis in these couples and refer them for karyotyping after two miscarriages to rule out the possible genetic cause of RPL, which can help further in proper prognostic assessment and genetic counselling of the concerned couple.

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