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Association of conventional chromosomal polymorphic variants in 580 couples with idiopathic recurrent pregnancy loss: A retrospective cohort study

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ABSTRACT

Objective: To investigate the potential link between chromosomal polymorphisms in couples who had a medical history of idiopathic recurrent pregnancy loss.

Methods: Cytogenetic investigation was conducted with mitogen (Phytohemagglutinin-M, Gibco) stimulated blood T lymphocytes by Giemsa trypsin Giemsa banding and Ag-NOR banding on 580 couples with a history of idiopathic recurrent pregnancy loss and 240 couples from the general population. Thirty good chromosomal spreads were captured, karyotyped, and analyzed. The karyotypes were designated using the International System for Human Cytogenomic Nomenclature 2024. Pearson *Chi*-square test was used to compare the frequency of chromosomal polymorphism variations in the idiopathic recurrent pregnancy loss group with the general population group.

Results: A conventional cytogenetic investigation revealed that 45.43% of couples experiencing idiopathic recurrent pregnancy loss presented with various types of chromosomal polymorphic variants, compared to 11.88% in the general population. The overall frequency of these chromosomal polymorphic variants was significantly higher in the idiopathic recurrent pregnancy loss group compared to the general population group (*OR* 9.97, 95% *CI* 6.99–14.21; *P*<0.05). Additionally, the prevalence of polymorphic variants was higher among males (49.14%) than females (41.72%) (*P*=0.01).

Conclusions: Chromosomal polymorphic analysis may play a crucial role in the assessment and careful clinical management of cases with idiopathic recurrent pregnancy loss, especially when no other conclusive reasons are identified during the initial evaluation. Therefore, heteromorphism should not be overlooked while investigating the causes of idiopathic recurrent pregnancy loss.

KEYWORDS: Polymorphic variants; Chromosomal anomalies; Repeated pregnancy loss; Cytogenetic investigation

Key Points

Question: Is the high prevalence of chromosomal polymorphic variants associated with cases of idiopathic recurrent pregnancy loss?

Results: In the current investigation, cytogenetic studies were performed on 1 160 individuals (580 couples) with a history of idiopathic recurrent pregnancy loss. The findings revealed that the frequency of chromosomal polymorphic variants was significantly higher in the idiopathic recurrent pregnancy loss group compared to the control group.

Meaning: Further molecular research is needed to investigate the potential link between chromosomally polymorphic couples experiencing idiopathic recurrent pregnancy loss, as this may play a crucial role in assessment and clinical management.

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1. Introduction

Recurrent pregnancy loss (RPL) is a prevalent medical condition, affecting around 1%-2% of reproductive-aged women around the world[1]. The American Society of Reproductive Medicine (ASRM) guidelines have defined RPL or habitual abortion as a medical condition characterized by two or more clinically failed pregnancies before 20 weeks of gestation[2]. According to Standard Treatment Guidelines for Management of Recurrent Spontaneous Abortions, 7.4% of Indian pregnancies result in repeated miscarriages, which is considered to be a very high incidence in nature. The results of scientific investigations had led to the development of national and international guidelines for the systemic assessment and precise management of couples with a history of idiopathic recurrent pregnancy loss (iRPL). Many known (50%) and unknown (50%) factors contribute to the aetiology of RPL, which includes uterine malformation or alternation (10%–15%), endocrinological diseases (17%–20%), autoimmune diseases (20%), infections (0.5%–5%), and chromosome abnormalities in couples (2%–5%)[3]. As per national and international guidelines about fifty percent of the patients may get the systemic assessment and precise management, and the other fifty percent are still classified as unknown (idiopathic), meaning that no significant, conclusive diagnosis can be made[4].

Various research indicates that numerical or structural anomalies, as well as polymorphic changes in either spouse, might result in reproductive failures[5–8]. In humans, euchromatin and heterochromatin are two structurally and functionally distinct regions of the chromosomes. Heterochromatin is extremely condensed nucleosomes, gene-poor, and transcriptionally inactive, whereas euchromatin is less condensed, contains unmethylated coding sequences, and is more readily transcribed. Chromosomal polymorphic variants are subtle variances of the tandem repeated sequences presented on the heterochromatin region[9]. The presence of these variations is due to the increase or decrease in the secondary constrictions on the q (long) arm of chromosomes 1, 9 and 16; alterations (addition/reduction) in the q (long) arm of the Y chromosome; and alterations in the p (short) arm containing the terminal part of the chromosomes beyond the secondary constrictions (satellites, stalks, and short arms) of chromosomes 13, 14, 15, 21, and 22[10]. Pericentric inversion of chromosome 9 is also considered a polymorphism[11].

Chromosomal polymorphic variants are attributed to occur in the general population as normal variants without any associated phenotypic or clinical impacts, but the true significance of chromosomal polymorphism or normal variation in human genetics is still unclear and under discussion[11]. Also, according to the International System for Human Cytogenomic Nomenclature (ISCN 2024), chromosome polymorphisms should not be included when reporting karyotype nomenclature. However, it can be included

in the report summary to prevent labelling errors and potential misinterpretation as a normal variant. Over the time, there has been substantial proofs, in addition to the analysis of whole chromosome anomalies, that variations in the p arm (short) and nucleolar organizing region (NOR), expansion or reductions in the secondary constriction on the q (long) arm, may be associated with infertility or reproductive failure in couples who experience RPL, infertility that remains unresolved, along with other reproductive failures[10,12–14].

Couples who have experienced repeated miscarriages are classified as idiopathic. It affects 1 in 300 pregnancies and is currently facing significant challenges due to their medical condition. Despite a standard RPL evaluation, which does not reveal any chromosomal aberrations, the medical expert finds that the patient's counselling is complicated and challenging. This study aims to look at the chromosomal aberrations and the potential relationship between chromosomal polymorphism and individuals with iRPL to improve their clinical management.

2. Methods

2.1. Study design and setting

The multicentric retrospective study was carried out in accordance with the Helsinki Declaration (2013) guidelines. The study was conducted from January 2018 to October 2024 at the School of Biotechnology, KIIT deemed to be University, in collaboration with inDNA Life Sciences Private Limited, Odisha, India. A detailed clinical history (family history, basal information, number of miscarriages, endocrine disorder, and the surgical and relevant diagnostic workup regarding reproductive aspects) and demographic details were obtained for each participant.

2.2. Participants

The study focused on 580 couples (1 160 individuals) with a history of iRPL, as well as 240 couples from the general population aged 19 to 58 years who sought treatment in the gynaecologic and obstetric department. These couples were part of fertility treatment. Those with incomplete clinical records and endocrine disorders were excluded from the study.

2.3. Chromosome preparation

A conventional 72-hours whole blood culture for lymphocytes was initiated with the help of RPMI-1640 containing 10%-12% of fetal bovine serum (Gibco-10270-106) along with 1% *L*-glutamine, 1% Penicillin Streptomycin (HiMedia-A007-100ML), and mitogen (Phytohemagglutinin-M-Gibco-10576-015). Mitotic cells were

harvested, and slides were prepared.

Slides were stained using the G-band by Giemsa trypsin Giemsa technique as per The AGT Cytogenetics Laboratory Manual, 4th edition.

2.3.1. Ag–NOR banding

As per the AGT Cytogenetics Laboratory Manual, 4th edition, Ag-NoR staining was performed to ascertain and confirm the presence of a constitutive heteromorphic region that contains tandem repeat sequences.

2.3.2. Chromosome analysis and image

With the help of the Olympus BX-43 compound microscope, 20 good-quality metaphase spreads were captured. Chromosomal analyses were carried out with the help of CytoVision 4.2 software and karyotyped as per the ISCN 2024 guideline.

2.4. Statistical analysis

All statistical analyses were conducted using IBM Corp.'s SPSS 29.0.2.0 software. The prevalence of chromosomal polymorphic variations as categorical variables was represented as percentages. The Pearson *Chi*-square test was performed to compare the frequency of chromosomal polymorphism variations in the group I (iRPL group) with the group II (general population). Chromosomal polymorphic variant frequency based on the gender distribution was also calculated. For sample sizes under five, Fisher's exact test was performed. Two separate analyses were carried out to compare the incidence of polymorphic variations between men and women in each group. Statistical significance was defined as $P < 0.05$.

2.5. Ethics statement

The research evaluation was conducted by an Independent Ethics Committee at inDNA Life Sciences Pvt. Ltd., Bhubaneswar, Odisha, and ethical approval was obtained (REF: IND/CYT/11/22/01, dated 10-11-2022). Before sample collection, voluntary informed consent was obtained from each patient in compliance with the standards set by the Ethics Committee. All personal information of every patient remained confidential.

3. Results

3.1. Association of structural and numerical chromosomal anomalies in iRPL individuals

In the current investigation, a cytogenetic analysis was performed on 1160 individuals (580 couples) with a clinical history of iRPL (male: 580 & female: 580). The findings revealed chromosomal

anomalies in 1.47% of the iRPL couples, which is within the limit of the percentage attributed to genetic factors that range around 1%-5% as per the published guidelines for assessment and medical care of couples experiencing RPL. This is also consistent with earlier reported investigations from the Indian subcontinent. Table 1 summarizes the study's overall outcome for the various kinds of structural and numerical chromosomal anomalies.

Out of 1160 cases in iRPL, 11 individuals were diagnosed with reciprocal translocation, 1 individual had a deletion, 1 individual had a duplication, 1 individual displayed an isochromosome, and 1 individual displayed three-way translocation. 3 individuals showed numerical chromosomal anomalies, and 2 individuals were presented with mosaicism, including numerical anomalies, and 2 were presented with mosaic reciprocal translocation. The most prevalent chromosomal abnormality discovered was structural chromosome abnormalities, which included reciprocal translocations in 0.95% of couples, accounting for about 1.47% of the total chromosome abnormalities reported in the research.

3.2. Association of chromosomal polymorphic variants in iRPL individuals

An additional investigation was performed and it showed presence of various types of chromosomal polymorphic variants like 1qh+, 9qh+, 16qh+, Yqh+, Yqh-, inversion of chromosome 9, inversion of chromosome Y, 13ps+, 14ps+, 15ps+, 21ps+, 22ps+, 13pstk+, 14pstk+, 15pstk+, 21pstk+ and 22pstk+ in iRPL individuals as illustrated in Figure 1.

Polymorphic variants were classified into different categories like qh+/- chromosomal polymorphic variants, qh+/- double chromosomal polymorphic variants, qh+/- triple chromosomal polymorphic variants, inversion of chromosome 9, inversion of chromosome Y, ps+/- chromosomal polymorphic variants, ps+/- double chromosomal polymorphic variants, ps+/- triple chromosomal polymorphic variants, ps+/- multiple chromosomal polymorphic variants, pstk+/- chromosomal polymorphic variants, pstk+/- double chromosomal polymorphic variants, pstk+/-, and multiple chromosomal polymorphic variants (Figure 2).

3.2.1. Association of chromosomal heteromorphic variants in iRPL group vs. general population

Further detailed examination revealed that the frequency of heterochromatin region 9qh+ (10.08%), Yqh+ (6.64%), 1qh+ (1.2%), 16qh+ (0.86%), and Yqh- (0.34%) was substantially greater as compared to the general population (group II). The frequency of inversion 9 (0.60%) and inversion Y (0.26%) polymorphic variants was also observed to be more prevalent in the iRPL group than the group II, as shown in Table 2 (A). The frequency of double and multiple chromosomal polymorphic variants of qh+ was also observed to be more prevalent in the iRPL group. The overall frequency of qh+/- chromosomal polymorphic variants, inversion of

Table 1. Chromosomal anomalies and polymorphic variants among 1 160 individuals (580 couples) with a history of idiopathic recurrent pregnancy loss [*n* (%)].

Cytogenetic outcome	Total (<i>n</i> =1160)	Male (<i>n</i> =580)	Female (<i>n</i> =580)
Normal karyotype (46,XY/46,XX)	616 (53.10)	289 (49.83)	327 (56.38)
Polymorphic variants	527 (45.43)	285 (49.14)	242 (41.72)
Chromosomal anomalies	17 (1.47)	6 (1.03)	11 (1.90)
Numerical anomalies	3 (0.26)	2 (0.34)	1 (0.17)
Non-mosaic numerical anomalies	1 (0.09)	1 (0.17)	0 (0.0)
Mosaic numerical anomalies	2 (0.17)	1 (0.17) 47,XYY	1 (0.17) 45,X/46,XX
Structural anomalies	14 (1.21)	4 (0.69)	10 (1.72)
Reciprocal translocation	11 (0.95)	4 (0.69)	7 (1.21)
Non-mosaic reciprocal translocation	9 (0.78)	2 (0.34)	7 (1.21)
Non-mosaic two-way reciprocal translocation	8 (0.69)	1 (0.17) 46,XY,t(11;22)(q24.1;q12.22)	7 (1.21) 46,XX,t(1;14)(p35;q31) 46,XX,t(2;4)(q36;q31.2) 46,XX,t(4;18)(p11;p11) 46,XX,t(5;6)(p13.3;q34) 46,XX,t(5;9)(q32.2;q32) 46,XX,t(8;18)(p23;q12) 46,X,t(X;3)(q23;p14)
Non-mosaic three-way reciprocal translocation	1 (0.09)	1 (0.17) 46,XY,t(4;7;16)(q31.1;q31.2;q32)	0 (0.00)
Mosaic reciprocal translocation	2 (0.17)	2 (0.34) 46,XY/46,XY,t(7;14)(p13.3;q34)	0 (0.00)
Isochromosome	1 (0.09)	0 (0.00)	1 (0.17) 46,X,i(X)(q10)
Deletion	1 (0.09)	0 (0.00)	1 (0.17) 46,X,der(X)del(X)(p11.3)del(X)(q21.3)-01
Duplication	1 (0.09)	0 (0.00)	1 (0.17)
Non-mosaic duplication	0 (0.00)	0 (0.00)	0 (0.00)
Mosaic duplication	1 (0.09)	0 (0.00)	1 (0.17)

chromosome 9, and Y was significantly higher ($P<0.05$) in the iRPL group with an odds ratio (OR) 95% confidential interval (CI) of 6.03 (3.77-9.65).

3.2.2. Association of chromosomal polymorphic variants in D/G (ps+) in iRPL group vs. general population

Further analysis revealed that the iRPL group had a significantly higher prevalence of polymorphism variants ps+ belonging to the D/G groups (17.93%) than the group II (general population) (5.62%). The frequency of 21ps+ subtype polymorphism (3.62%) was considerably greater ($P=0.004$) in the acro ps+ group, with OR (95% CI) of 3.57 (1.40-9.08). The frequency of 13ps+, 14ps+, 22ps+, double ps+, triple ps+, and multiple ps+ polymorphic variants was also observed to be more prevalent in the iRPL group than the group II, as shown in Table 2 (B). The overall frequency of ps+, ps+ double, and ps+ multiple chromosomal polymorphic variants was significantly higher ($P<0.05$) in the iRPL group with OR (95% CI) of 3.67 (2.42-5.56).

3.2.3. Association of chromosomal polymorphic variants in D/G (pstk+) in iRPL group vs. general population

The analysis revealed that the iRPL group had a higher prevalence of polymorphism variants pstk+ belonging to the D/G groups (4.22%) than the group II (general population) (2.08%). The frequency of

21pstk+ (1.21%) and 22pstk+ (1.03%) subtype polymorphism was higher in the acro pstk+ group compared to the group II, whereas the frequency of 13pstk+ (0.26%), 14pstk+ (0.60%) and 15pstk+ (0.60%) were comparatively higher compared to the group II. The overall frequency of pstk+, and pstk+ double chromosomal polymorphic variants was significantly higher ($P=0.03$) in the iRPL group with OR (95% CI) of 2.07 (1.04-4.13) shown in Table 2 (C).

3.2.4. Association of multiple chromosomal polymorphic variants in iRPL group vs. general population

Multiple polymorphic variants were also observed to be more prevalent in the iRPL group (2.50%) than the group II (general population), as shown in Table 2 (D). The overall frequency of multiple chromosomal polymorphic variants was significantly higher ($P<0.05$) in the iRPL group with OR (95% CI) of 9.97 (6.99-14.21).

3.3. Association of chromosomal polymorphic variants based on the gender distribution

The frequency of polymorphic variants was further analyzed based on the gender distribution (Figure 3) and was significantly higher ($P<0.05$) in the male iRPL group compared to the female iRPL group (Table 3).

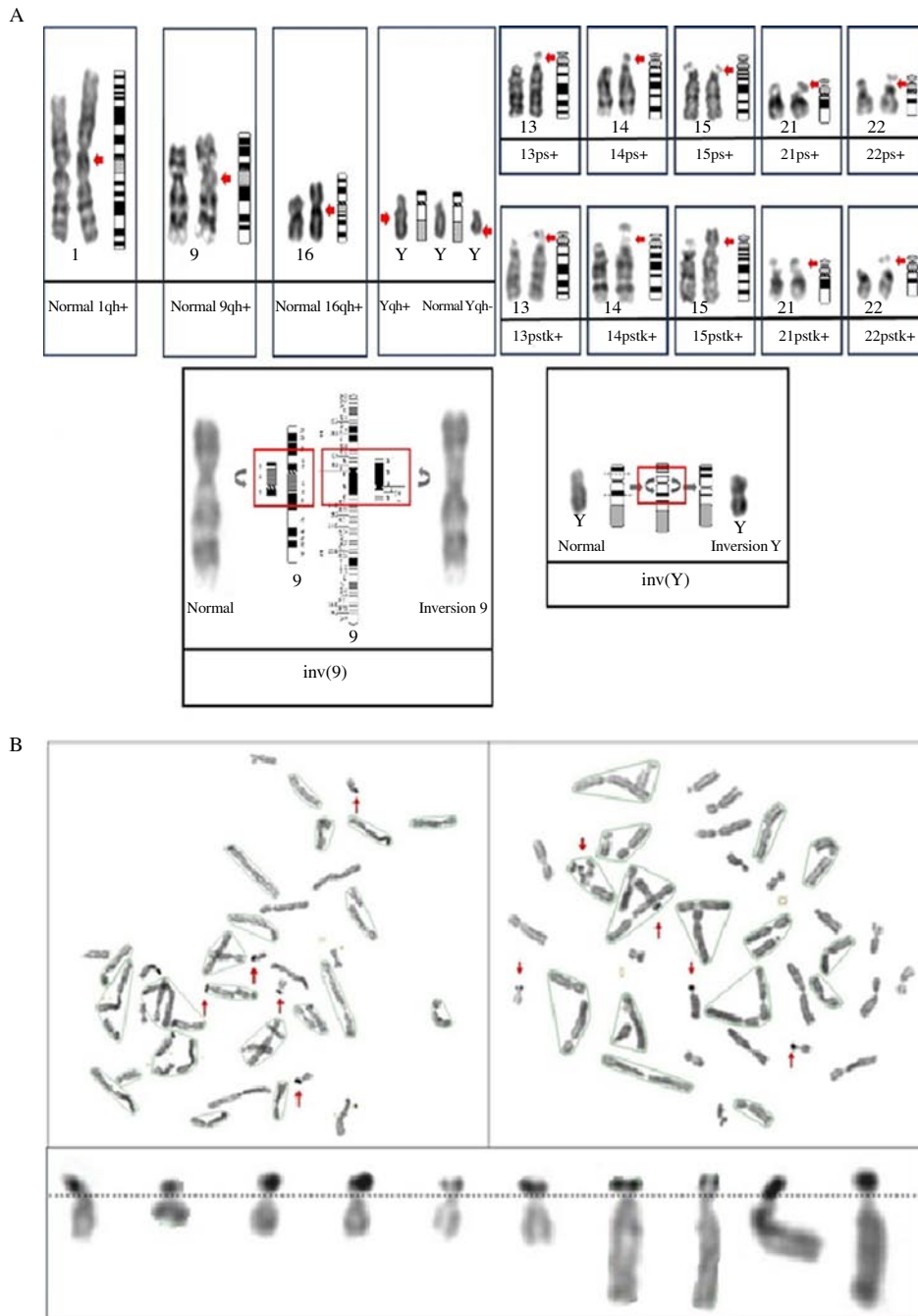


Figure 1. A) Representative images of chromosomal variants in the iRPL group. **B)** Representative karyotype (iRPL individual) showing a chromosomal variant on the NOR region of the acrocentric chromosome's short p-arm (red arrows). iRPL: idiopathic recurrent pregnancy loss; NOR: nucleolar organizing region.

3.3.1. Association of chromosomal heteromorphic variants based on the gender distribution

The prevalence of the 9qh+ variant was significantly higher in the female group (10.52%) as compared to the male group (9.66%). The overall frequency of heteromorphic variants was significantly higher ($P < 0.05$) in iRPL male (28.28%) compared to iRPL female (13.28%) [Table 3 (A)].

The frequency of qh+/- polymorphic variants was further analyzed based on the gender distribution, and it was found that the prevalence of overall polymorphic variants was significantly higher ($P = 0.01$) in males (49.14%) as compared to females (41.72%), as shown in Table 3 (Grand total A+B+C+D).

3.3.2. Association of D/G groups chromosomal polymorphic variants based on the gender distribution

The female group had high a prevalence ($P < 0.001$) of polymorphism variants ps+ belonging to the D/G groups (22.07%) than the male group (13.79%) [Table 3 (B)]. Further analysis revealed that prevalence of 13ps+ (1.90%), 15ps+ (3.45%), 21ps+ (5.00%), ps+ double chromosomal polymorphic variants like 14ps+ & 21 ps+ (0.52%), 14ps+ & 22 ps+ (0.86%), 15ps+ & 21 ps+ (0.17%), 21ps+ & 22 ps+ (1.03%), triple chromosomal polymorphic variant like 13ps+, 14ps+ & 15 ps+ (0.86%) and multiple chromosomal polymorphic variant like 13ps+, 14ps+, 15ps+, 21ps+, 22ps+ (4.31%) variants were significantly higher in the female group as compared

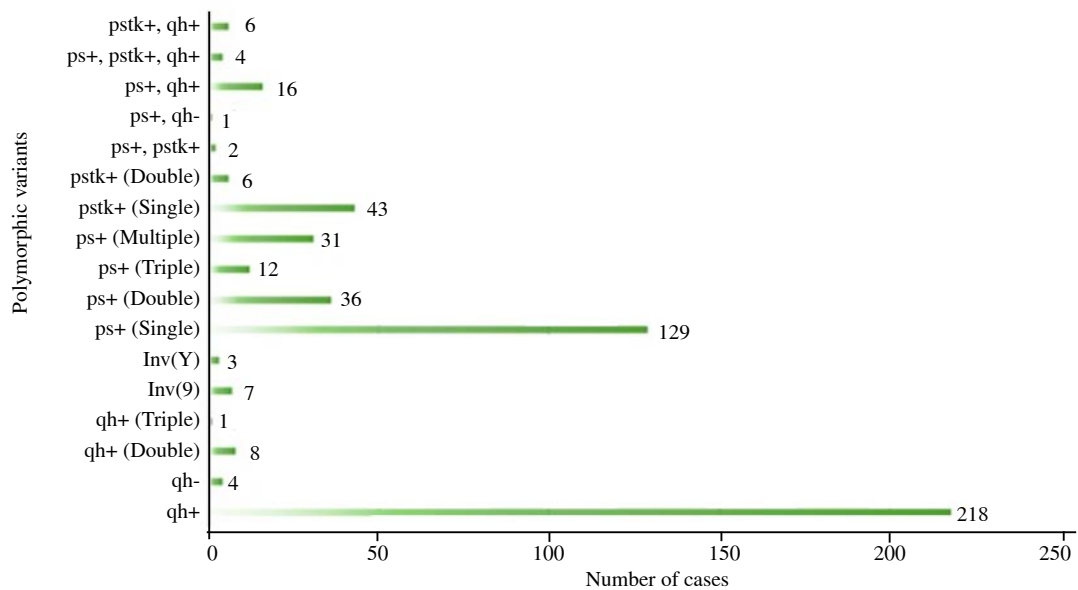


Figure 2. Number of various types of chromosomal polymorphic variants observed in group I (iRPL individuals). iRPL: idiopathic recurrent pregnancy loss.

Table 2. Distribution and comparison of chromosomal polymorphic variants in group I (iRPL group) and II (general population who had no history of RPL) [n (%)].

Types of variant	Chromosomal polymorphism	Group I (n=1160)	Group II (n=480)	OR (95% CI)	P-value
A. Chromosomal heteromorphic variants					
qh+ (n=218)	1qh+	14 (1.2)	0 (0.00)	Ref	0.02
	9qh+	117 (10.08)	11 (2.29)	4.78 (2.55-8.96)	<0.05
	16qh+	10 (0.86)	2 (0.42)	2.08 (0.45-9.52)	0.34
	Yqh+	77 (6.64)	4 (0.83)	8.46(3.08-23.25)	<0.05
qh- (n=4)	Yqh-	4 (0.34)	0 (0.00)	Ref	0.20
qh+ (double) (n=8)	9,Y	4 (0.34)	1 (0.21)	1.66 (0.18-14.87)	0.65
	9,16	2 (0.17)	0 (0.00)	Ref	0.36
	1,9	1 (0.09)	0 (0.00)	Ref	0.52
	1,Y	1 (0.09)	0 (0.00)	Ref	0.52
qh+ (triple) (n=1)	1,9,Y	1 (0.09)	0 (0.00)	Ref	0.52
Inversions (n=10)	Inv(9)	7 (0.60)	1 (0.21)	2.91 (0.36-23.70)	0.30
	Inv(Y)	3 (0.26)	1 (0.21)	1.24 (0.13-11.97)	0.85
Total		241 (20.77)	20 (4.17)	6.03 (3.77-9.65)	<0.05
B. Chromosomal polymorphic variants in D/G (ps+)					
ps+ (single) (n=129)	13ps+	20 (1.72)	4 (0.83)	2.09 (0.71-6.14)	0.17
	14ps+	20 (1.72)	3 (0.63)	2.79 (0.83-9.43)	0.09
	15ps+	33 (2.84)	1 (0.21)	14.03 (1.91-102.85)	<0.001
	21ps+	42 (3.62)	5 (1.04)	3.57 (1.40-9.08)	0.004
	22ps+	14 (1.21)	2 (0.42)	2.92 (0.66-12.90)	0.14
Ps+ (double) (n=36)	14,21	4 (0.34)	2 (0.42)	0.83 (0.15-4.53)	0.83
	15,21	1 (0.09)	0 (0.00)	Ref	0.52
	21,22	10 (0.86)	2 (0.42)	2.08 (0.45-9.52)	0.34
	13,14	5 (0.43)	1 (0.21)	2.07 (0.24-17.80)	0.50
	13,21	2 (0.17)	0 (0.00)	Ref	0.36
	14,15	1 (0.09)	0 (0.00)	Ref	0.52
	13,15	4 (0.34)	2 (0.42)	0.83 (0.15-4.53)	0.42
	13,22	1 (0.09)	1 (0.21)	0.41 (0.03-6.62)	0.52
14,22	8 (0.69)	0 (0.00)	Ref	0.07	
Ps+ (triple) (n=12)	14,15,22	1 (0.09)	0 (0.00)	Ref	0.52
	14,15,21	3 (0.26)	1 (0.21)	1.24 (0.13-11.97)	0.85
	13,15,21	1 (0.09)	0 (0.00)	Ref	0.52
	13,14,15	7 (0.60)	1 (0.21)	2.91 (0.36-23.70)	0.30
Ps+ (multiple) (n=31)	13,14,15,21,22	31 (2.67)	2 (0.42)	6.56 (1.56-27.53)	0.003
Total		208 (17.93)	27 (5.62)	3.67 (2.42-5.56)	<0.05

Table 2. Continued.

Types of variant	Chromosomal polymorphism	Group I (n=1160)	Group II (n=480)	OR (95% CI)	P-value
C. Chromosomal polymorphic variants in D/G groups (pstk+)					
Pstk+ (single) (n=43)	13	3 (0.26)	1 (0.21)	1.24 (0.13-11.97)	0.85
	14	7 (0.60)	1 (0.21)	2.91 (0.36-23.70)	0.30
	15	7 (0.60)	3 (0.63)	0.97 (0.25-3.75)	0.96
	21	14 (1.21)	3 (0.63)	1.94 (0.56-6.79)	0.29
	22	12 (1.03)	2 (0.42)	2.50 (0.56-11.21)	0.22
Pstk+ (double) (n=6)	13,14	1 (0.09)	0 (0.00)	Ref	0.52
	13,21	1 (0.09)	0 (0.00)	Ref	0.52
	13,15	1 (0.09)	0 (0.00)	Ref	0.52
	13,22	1 (0.09)	0 (0.00)	Ref	0.52
	21,22	1 (0.09)	0 (0.00)	Ref	0.52
	14,21	1 (0.09)	0 (0.00)	Ref	0.52
Total		49 (4.22)	10 (2.08)	2.07 (1.04-4.13)	0.03
D. Multiple chromosomal polymorphic variants					
(Ps+)+(Pstk+) (n=2)	(21,22)+(14)	2 (0.17)	0 (0.00)	Ref	0.36
(Ps+)+(qh-) (n=1)	(13,14,15,21,22)+(Y)	1 (0.09)	0 (0.00)	Ref	0.52
(Pstk+)+(qh+) (n=6)	(14)+(9)	2 (0.17)	0 (0.00)	Ref	0.36
	(14)+(1)	1 (0.09)	0 (0.00)	Ref	0.52
	(21)+(9)	1 (0.09)	0 (0.00)	Ref	0.52
	(15,21)+(9)	1 (0.09)	0 (0.00)	Ref	0.52
	(13,15,21)+(Y)	1 (0.09)	0 (0.00)	Ref	0.52
(Ps+)+(qh+) (n=16)	(13)+(Y)	1 (0.09)	0 (0.00)	Ref	0.52
	(14)+(Y)	1 (0.09)	0 (0.00)	Ref	0.52
	(14)+(9)	1 (0.09)	0 (0.00)	Ref	0.52
	(15)+(Y)	1 (0.09)	0 (0.00)	Ref	0.52
	(15)+(16)	1 (0.09)	0 (0.00)	Ref	0.52
	(21)+(9)	2 (0.17)	0 (0.00)	Ref	0.36
	(14,15)+(9)	1 (0.09)	0 (0.00)	Ref	0.52
	(14,15)+(Y)	1 (0.09)	0 (0.00)	Ref	0.52
	(13,21)+(9)	1 (0.09)	0 (0.00)	Ref	0.52
	(14,21)+(9)	2 (0.17)	0 (0.00)	Ref	0.36
	(14,21)+(Y)	1 (0.09)	0 (0.00)	Ref	0.52
	(15,22)+(Y)	1 (0.09)	0 (0.00)	Ref	0.52
	(14,15,22)+(9)	1 (0.09)	0 (0.00)	Ref	0.52
(13,14,15,21,22)+(Y,16)	1 (0.09)	0 (0.00)	Ref	0.52	
(Ps+) + (Pstk+)+(qh+) (n=4)	(22)+(14)+(9)	2 (0.17)	0 (0.00)	Ref	0.36
	(13,21)+(22)+(9)	2 (0.17)	0 (0.00)	Ref	0.36
Total		29 (2.50)	0 (0.00)	Ref	<0.001
Grand Total A+B+C+D		527 (45.43)	57 (11.88)	9.97 (6.99-14.21)	<0.05

Chi square or Fisher's exact test (if any frequency <5) is used. OR: odds ratio; CI: confidence interval. RPL: recurrent pregnancy loss; iRPL: idiopathic recurrent pregnancy loss; Ref: reference.

to the male group [Table 3 (B)].

The analysis revealed that the female group had a higher prevalence of polymorphism variants pstk+/- belonging to the D/G groups (4.83%) than the male group (3.62%) [Table 3 (C)].

The frequency of 14pstk+ (0.86%), 21pstk+ (1.38%), 22pstk+ (1.38%), double chromosomal polymorphic variants like 13stk+,15pstk+ (0.17%),13pstk+,22pstk+ (0.17%) and 21pstk+,22pstk+ (0.17%) subtype polymorphisms were higher in the acro pstk+ group in female as compared to the male group, whereas the frequency of 13pstk+ (0.17%), 15pstk+ (0.52%), double chromosomal polymorphic variants like 13pstk+,14pstk+ (0.00%), 13pstk+,21pstk+ (0.00%) and 14pstk+,21pstk+ (0.00%) was lower

in the female group as compared to the male group [Table 3 (C)].

3.3.3. Association of multiple chromosomal polymorphic variants based on the gender distribution

Apart from single, double, and triple chromosomal polymorphic variants, multiple chromosomal polymorphic variants of D/G groups, along with heteromorphic variants, were also observed in multiple cases, as shown in Table 3 (D).

The analysis revealed that the male group (3.45%) had a higher prevalence of multiple chromosomal polymorphic variants of D/G groups, along with heteromorphic variants, than the female group (1.55%) ($P=0.04$) [Table 3 (D)].

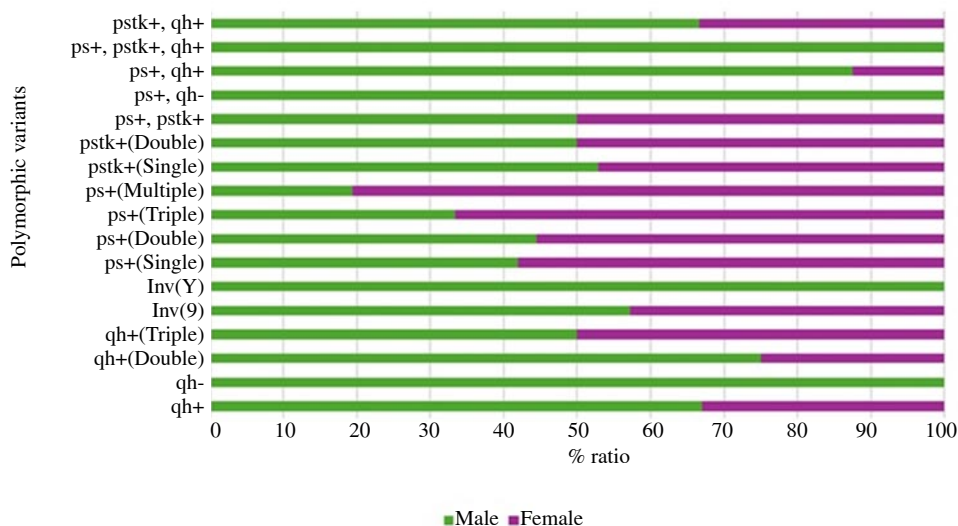


Figure 3. Distribution of chromosomal polymorphisms in males and females.

Table 3. Descriptions and prevalence of several chromosomal polymorphism variations identified in males and females (580 couples) with a history of idiopathic recurrent pregnancy loss [n (%)].

Types of variant	Chromosomal polymorphism	Male (n=580)	Female (n=580)	Total (n=1160)	P-value
A. Heteromorphic variants					
qh+ (n=218)	1qh+	5 (0.86)	9 (1.55)	14 (1.21)	0.28
	9qh+	56 (9.66)	61 (10.52)	117 (10.09)	0.63
	16qh+	8 (1.38)	2 (0.34)	10 (0.86)	0.06
	Yqh+	77 (13.27)	0 (0.00)	77 (6.64)	--
qh- (n=4)	Yqh-	4 (0.69)	0 (0.00)	4 (0.35)	--
qh+ (double) (n=8)	9,Y	4 (0.69)	0 (0.00)	4 (0.35)	--
	9,16	1 (0.17)	1 (0.17)	2 (0.17)	>0.99
	1,9	0 (0.00)	1 (0.17)	1 (0.09)	--
	1,Y	1 (0.17)	0 (0.00)	1 (0.09)	--
qh+ (triple) (n=1)	1,9,Y	1 (0.17)	0 (0.00)	1 (0.09)	--
Inversions (n=10)	Inv(9)	4 (0.69)	3 (0.52)	7 (0.60)	0.70
	Inv(Y)	3 (0.52)	0 (0.00)	3 (0.26)	--
Total		164 (28.28)	77 (13.28)	241 (20.77)	<0.05
B. Polymorphic variants in D/G groups (ps+)					
ps+ (single) (n=129)	13ps+	9 (1.55)	11 (1.90)	20 (1.72)	0.65
	14ps+	11 (1.90)	9 (1.55)	20 (1.72)	0.65
	15ps+	13 (2.24)	20 (3.45)	33 (2.84)	0.22
	21ps+	13 (2.24)	29 (5.00)	42 (3.62)	0.01
	22ps+	8 (1.38)	6 (1.03)	14 (1.21)	0.59
Ps+ (double) (n=36)	14,21	1 (0.17)	3 (0.52)	4 (0.34)	0.32
	15,21	0 (0.00)	1 (0.17)	1 (0.09)	0.32
	21,22	4 (0.69)	6 (1.03)	10 (0.86)	0.53
	13,14	3 (0.52)	2 (0.34)	5 (0.43)	0.65
	13,21	2 (0.34)	0 (0.00)	2 (0.17)	<0.001
	14,15	1 (0.17)	0 (0.00)	1 (0.09)	0.32
	13,15	2 (0.34)	2 (0.34)	4 (0.34)	>0.99
	13,22	0 (0.00)	1 (0.17)	1 (0.09)	0.32
14,22	3 (0.52)	5 (0.86)	8 (0.69)	0.48	
Ps+ (triple) (n=12)	14,15,22	0 (0.00)	1 (0.17)	1 (0.09)	0.32
	14,15,21	2 (0.34)	1 (0.17)	3 (0.26)	0.56
	13,15,21	2 (0.34)	5 (0.86)	7 (0.60)	0.26
	13,14,15	0 (0.00)	1 (0.17)	1 (0.09)	0.32
Ps+ (multiple) (n=31)	13,14,15,21,22	6 (1.03)	25 (4.31)	31 (2.67)	<0.001
Total		80 (13.79)	128 (22.07)	208 (17.93)	<0.001

Table 3. Continued.

Types of variant	Chromosomal polymorphism	Male (n=580)	Female (n=580)	Total (n=1160)	P-value
C. Polymorphic variants in D/G groups (pstk+)					
Pstk+ (single) (n=43)	13	2 (0.34)	1 (0.17)	3 (0.26)	0.56
	14	2 (0.34)	5 (0.86)	7 (0.60)	0.26
	15	4 (0.69)	3 (0.52)	7 (0.60)	0.70
	21	6 (1.03)	8 (1.38)	14 (1.21)	0.59
	22	4 (0.69)	8 (1.38)	12 (1.03)	0.25
Pstk+ (double) (n=6)	13,14	1 (0.17)	0 (0.00)	1 (0.09)	0.32
	13,21	1 (0.17)	0 (0.00)	1 (0.09)	0.32
	13,15	0 (0.00)	1 (0.17)	1 (0.09)	0.32
	13,22	0 (0.00)	1 (0.17)	1 (0.09)	0.32
	21,22	0 (0.00)	1 (0.17)	1 (0.09)	0.32
	14,21	1 (0.17)	0 (0.00)	1 (0.09)	0.32
Total		21 (3.62)	28 (4.83)	49 (4.22)	0.31
D. Presence of multiple chromosomal polymorphic variants					
(Ps+)+(Pstk+) (n=2)	(21,22)+(14)	1 (0.17)	1 (0.17)	2 (0.17)	0.09
(Ps+)+(qh-) (n=1)	(13,14,15,21,22)+(Y)	1 (0.17)	0 (0.00)	1 (0.09)	--
(Pstk+)+(qh+) (n=6)	(14)+(9)	1 (0.17)	1 (0.17)	2 (0.17)	>0.99
	(14)+(1)	1 (0.17)	0 (0.00)	1 (0.09)	0.32
	(21)+(9)	0 (0.00)	1 (0.17)	1 (0.09)	0.32
	(15,21)+(9)	1 (0.17)	0 (0.00)	1 (0.09)	0.32
	(13,15,21)+(Y)	1 (0.17)	0 (0.00)	1 (0.09)	--
(Ps+)+(qh+) (n=16)	(13)+(Y)	1 (0.17)	0 (0.00)	1 (0.09)	--
	(14)+(Y)	1 (0.17)	0 (0.00)	1 (0.09)	--
	(14)+(9)	0 (0.00)	1 (0.17)	1 (0.09)	0.32
	(15)+(Y)	1 (0.17)	0 (0.00)	1 (0.09)	--
	(15)+(16)	0 (0.00)	1 (0.17)	1 (0.09)	0.32
	(21)+(9)	2 (0.34)	0 (0.00)	2 (0.17)	<0.001
	(14,15)+(9)	1 (0.17)	0 (0.00)	1 (0.09)	0.32
	(14,15)+(Y)	1 (0.17)	0 (0.00)	1 (0.09)	--
	(13,21)+(9)	1 (0.17)	0 (0.00)	1 (0.09)	0.32
	(14,21)+(9)	2 (0.34)	0 (0.00)	2 (0.17)	<0.001
	(14,21)+(Y)	1 (0.17)	0 (0.00)	1 (0.09)	--
	(15,22)+(Y)	1 (0.17)	0 (0.00)	1 (0.09)	--
	(14,15,22)+(9)	1 (0.17)	0 (0.00)	1 (0.09)	0.32
(13,14,15,21,22)+(Y,16)	1 (0.17)	0 (0.00)	1 (0.09)	--	
(Ps+)+(Pstk+)+(qh+) (n=4)	(22)+(14)+(9)	0 (0.00)	2 (0.34)	2 (0.17)	0.16
	(13,21)+(22)+(9)	0 (0.00)	2 (0.34)	2 (0.17)	0.16
Total		20 (3.45)	9 (1.55)	29 (2.50)	0.04
Grand Total A+B+C+D		285 (49.14)	242 (41.72)	527 (45.43)	0.01

Chi square or Fisher's exact test (if any frequency <5) is used.

4. Discussion

Giemsa trypsin Giemsa (GTG)-banding detects frequently observed cytogenetic polymorphic variants, which are called heteromorphisms in the general population. The relationship between chromosomal polymorphic variants and human reproductive biology is an area of interest to be elucidated. Over the years, various studies around the world have revealed a much higher incidence of chromosomal polymorphisms at intervals of 8%-22% among sub-fertile couples compared to fertile ones[14,21-24]. In contrast, in the current study, we identify a greater prevalence of chromosomal polymorphism rather than numerical and structural anomalies, around 45.43%, which is

much more prevalent among any population.

Heteropolymorphic variants are common on the long arm of chromosomes 1, 9, 16, and Y, as well as significant acrocentric short arms in the form of satellites and stalks on chromosomes 13, 14, 15, 21, and 22[25]. Increased heterochromatin area on a long arm (q) of chromosome 1, 9, 16, and Y is denoted as qh+, whereas acrocentric chromosomes with longer short arm satellites and stalks are referred to as ps+ and pstk+, respectively[26]. The nucleotide sequence of chromosomes 1, 9, and 16 reveals the biggest autosomal region of heterochromatin, which is highly individualized in 6%-8% of persons[15-21,27]. In the present investigation, we observed that the variations in heterochromatin on chromosomes 1, 9, 16, and Y were

significantly higher compared to previous research. Specifically, chromosome 9 had the most polymorphisms, with females having a higher number of variations than males.

Rearrangements caused by two or more breakpoints on a single chromosome, which result in the inversion and reinsertion of a segment, are known as a chromosomal inversion event. There are two forms of inversions: pericentric, which contains the chromosome's centromere, and paracentric, which excludes it. A balanced pericentric inversion often has no clinical repercussions for its carrier and occurs in 1%-2% of the overall population. Nevertheless, throughout meiosis, there is a possibility of inversion loop creation. An unequal amount of crossing-over events inside the inversion loop might result in imbalanced offspring by *de novo* deletion, duplication, or in combination. Chromosome 9 inversion is a frequent structural balanced chromosomal abnormality, with an estimated prevalence of 3.5%. Numerous instances have been linked to congenital abnormalities, growth retardation, infertility, repeated pregnancy loss, and malignancy[28]. Variations of pericentric inversion in chromosome 9 were identified to be substantially more common in males with idiopathic reproductive failure than in females.

The link between Y chromosomal polymorphic variations and idiopathic reproductive failure remains debatable. According to various research, modifications in the long arm(q) of the Y chromosome resulted in altered transcription, control of functional transcription elements like heat shock transcription component, and spermatogenesis-related regions like azoospermia factor (AZF).

According to a few studies, pericentric inversion of the human Y chromosome is an uncommon heteromorphism without any clinical consequence. The link between Y chromosomal polymorphic variations and iRPL should be regarded with caution, attributable to the substantial heterogeneity of male individuals, which can be acquired by male offspring, with ambiguous effects on reproductive results[29]. However, Sheth *et al* found that inverted Y chromosomes do not impair sperm production and, like normal Y chromosomes, are inherited with little medical importance[30].

The limited number of investigations in the field of cytogenetics demonstrated that a decrease (Yqh-) in heterochromatin in the long arm of the Y chromosome might lead to mitotic errors, leading to stillbirth or recurrent pregnancy loss[31].

As per various investigations, the heterochromatin region found in the centromere area is crucial for spindle attachment, chromosomal mobility, meiotic pairing, and sister chromatid cohesion. Modifications in D/G group chromosome variant regions can disrupt kinetochore assemblage and centromere functioning, make homologous chromosome pairing challenging, negatively impact cell division, and hinder gamete formation[32]. The hypothesis recommends that the presence of chromosomal polymorphisms in a population should be evenly distributed and stable. In contrast to the

hypothesis, we found that iRPL individuals had significantly higher prevalence of chromosomal polymorphisms when compared with prior published research from all over the world[1,5].

Until recently, heterochromatin was assumed to be 'junk' or inactive DNA[9,33,34]. It is now accepted that heterochromatin characteristics and molecular makeup are comparable to those of segments of chromosomes associated with suppression of genes, location impact variation, and inactivation of X chromosomes[35]. The development of a certain type of chromatin packing induced by epigenetic indicators in DNA, such as gene promoter hypermethylation and histone acetylation, is required for regulating gene expression and silencing[36]. The occurrence of chromosomal variations in men and women suggests the presence of extensive heterochromatin blocks that are accountable for chromosome pairing, deteriorating spindle fiber attachment, or downregulation of active genes, resulting in meiotic arrest in gametogenesis and sterility[22]. Furthermore, acrocentric and paracentric chromosomes include a NOR, satellite stalks, and satellites with heterochromatin[37]. According to various studies, heterochromatin variants are influenced by temperature stimuli such as heat shock and heat shock protein (HSP) assembly, as well as epigenetic signals, which have a role in gene regulation and control, impacting reproductive function in both situations[38,39].

The current study has several limitations. Firstly, the detailed demographic characteristics of the participants were inadequate, and we could not follow up on their subsequent health status. Secondly, some subtle duplications and deletions remained undetected due to the limited resolution of traditional G-banding. Moreover, while fluorescence in situ hybridization (FISH) and microarray investigations are not routinely performed in instances of reproductive failure, we cannot exclude the possibility of cryptic translocations, microdeletions, and microduplications.

In conclusion, the presence or absence of chromosomal polymorphic variants in RPL couples should be evaluated with caution, as their presence might be a potential contributor to prognosis and clinical management. Cases positive for presence of chromosomal polymorphic variant need evaluation with through addition cytogenetic and molecular assays to ascertain their role, and should be followed on a case-by-case basis until more robust scientific proof is available. Meanwhile, routine cytogenetic investigation needs careful detailing, which can assist treating physicians in consultation and prompt therapeutic intervention to prepare for upcoming pregnancies in aspiring couples classified with iRPL. The present study found that Indian RPL couples had a significantly high prevalence rate of chromosomal polymorphisms than the control group, which raises the need for additional research at molecular level to look into the potential connection between chromosomally polymorphic couples who experience iRPL.

Conflict of interest statement

The authors have no competing interests to declare.

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Authors' contributions

Sunny Kumar Jignesh Kumar Patel participated in conception of the work, drafting the article, data analysis and interpretation; Amit Roy Chowdhury participated in critical revision of the article and final approval for publication; Shagufa Sheikh performed conventional cytogenetic technique and data curation; Purna Chandra Mahapatra contributed to investigation, data acquisition, and validation; Birendranath Banerjee participated in critical revision of the article and final approval for publication. All authors read and approved the final manuscript.

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