DOI: https://doi.org/10.52756/ijerr.2024.v37spl.007 *Int. J. Exp. Res. Rev.*, Vol. 36: 85-95 (2024)

Original Article | *Peer Reviewed* | *Open Access*

International Journal of Experimental Research and Review (IJERR)

ISSN: 2455-4855 (Online)
www.iaph.in
ISSN 2455-4855

Recurrent Pregnancy Loss Associated Cytogenetic and Genetic Anomalies – Study from Eastern India

Koutilya Bhattacharjee[1]*, Suman Pal[1], Emili Banerjee[2] and Shiuli Mukherjee[3]

[1]Department of Zoology, Ramakrishna Mission Vivekananda Centenary College, West Bengal, India; [2]Geneticist and Genetic Counselor, MFC Woman and Child Care, West Bengal, India; Neuberg Center for Genomic Medicine, Neuberg Diagnostics, Gujrat, India; [3]Gynecologist and Proprietor, Mukherjee Fertility Centre, West Bengal, India.

E-mail/Orcid Id:

KB, sendtokoutilya@gmail.com, https://orcid.org/0000-0002-6752-4840; *SP,* sumanpal98@gmail.com, https://orcid.org/0009-0009-3774-6694; *EB,* dremili.geneticist@gmail.com, https://orcid.org/0000-0003-4432-6996; *SM,* shiuli.mukherjee@gmail.com, https://orcid.org/0009-0001-5814-3392

Article History:

Received: 20th Dec., 2023
Accepted: 09th Mar., 2024
Published: 30th Mar., 2024

Keywords:
REPL, 9Qh+, chromosomal heteromorphy, karyotype, compound heterozygosity, secondary infertility

How to cite this Article:
Yasodha Kesavan, Sheik Mohideen Sahabudeen and Satish Ramalingam (2023). Exosomes derived from metastatic colon cancer cells induced oncogenic transformation and migratory potential of immortalized human cells. *International Journal of Experimental Research and Review*, *37*(Spl.), 85-95.
DOI:
https://doi.org/10.52756/ijerr.2024.v37spl.007

Abstract: Recurrent early pregnancy loss (REPL) refers to loss (2 or more) of pregnancy within the first trimester of the gestation period. Reports of REPL cases are significantly increasing along with idiopathic or enigmatic REPL because associated clinical parameters in such cases remain within normal range and diagnosis remains odyssey. Genetic factors like single nucleotide variations (SNV) and altered heterochromatin and/or satellite content, as chromosomal aberrations like translocation, deletion and inversions have reportedly remained associated with many diseases. The present study aimed to find any structural and single nucleotide variations associated with idiopathic REPL. Three thousand six hundred twelve (3612) couples with history of 2 or more pregnancy losses or neonatal death were subjected to clinical investigations, karyotype analyses and Whole Exome Sequence analyses as per requirement to identify the underlying cause(s). More than 14% of the idiopathic REPL cases were found to carry chromosomal heteromorphisms. Among these, 9qh+ was predominant, followed by 21ps+, 15ps+, 14ps+ and others. Heteromorphies were significantly higher in females than males except for 14ps+. Along with some single nucleotide variations were also found among the subjects, though compound heterozygosity or allelic homozygosity were major causal factors. Idiopathic REPL cases were found to carry genetic variations, as per the present study, with a prevalence of more than 10% among RPL cases with yet unknown molecular mechanisms of damage. A further thorough study to unveil the underlying molecular pathology is strongly recommended.

Introduction

Recurrent Early Pregnancy Loss (REPL) is a combinatorial situation of two pregnancy-related problems, i.e., recurrent pregnancy loss (RPL) and early pregnancy loss (EPL). According to the guidelines of National Institute for Health and Clinical Excellence (NCCWCH UK Guidelines, 2012), a pregnancy loss within 13 weeks of gestation with foetal weight below 500 grams is termed as EPL, while two or more intra-uterine foetal demise (IUFD) will be called as RPL. Therefore, an experience of two or more pregnancy losses within 13 weeks of gestation for each time could be termed as REPL. REPL has a reported global prevalence of 0.7 to 1.9% (Quenby et al., 2021), with a similar reported statistical range of 1 to 2% in the Indian population (Dhaded et al., 2018) of all reported pregnancies. Miscarriages, be it early or late, always result in trauma to the family along with the experiencing couple with a lot of social impacts. Such mental agony affects not only socially but also since the would-be parents primarily contribute to the workforce of any country. Therefore, the impact also compromises human resources, yielding economic damage to society. RPL has several contributing aetiological factors, viz., lifestyle

***Corresponding Author:** sendtokoutilya@gmail.com

Table 1. Comprehensive classification of common causal factors related to RPL (including REPL)

Causal Factor	Details
Anatomical Abnormalities	➢ Submucosal fibroids ➢ Septate uterus ➢ Bicornuate uterus ➢ Unicornuate uterus ➢ Incompetent Cervix
Sperm Pathologies	➢ Polyzoospermia – sperm concentration > 200 million/ml ➢ Teratozoospermia – morphological defects of sperms ➢ Sperm DNA fragmentation
Endocrine complexities	➢ PCOS ➢ Obesity ➢ Insulin related disorders ➢ Thyroid hormone related disorders ➢ LH related disorders ➢ Androgen hypersecretion ➢ Hyposecretion of hCG ➢ Below normal level of AMH
Autoimmunity	➢ Antiphospholipid Syndrome ➢ Undifferentiated Connective Tissue Disease ➢ Systemic Lupus Erythematosus
Infections	➢ *Toxoplasma gondii* ➢ Rubella virus ➢ Cytomegalovirus ➢ Herpes simplex virus ➢ Tubercular and non-tubercular *Mycobacterium* ➢ *Neisseria gonorrhoeae* ➢ *Chlamydia trachomatis* ➢ *Mycoplasma genitalium* ➢ *Mycoplasma hominis* ➢ *Ureaplasma urealyticum* ➢ *Streptococcus agalactiae* (Group B) ➢ *Staphylococcus aureus*
Other major causes	➢ Thrombophilia ➢ Maternal Vitamin D deficiency
Lifestyle factors	➢ Smoking ➢ Excessive alcohol intake ➢ Exposure to environmental pollutants and toxins
Enigmatic / Idiopathic	???

habits, sperm pathologies, structural abnormalities of the uterus, endocrine complexities, autoimmunity, infections and last but not the least, idiopathic (since they lack any clinical diagnosis). Based on the available literature, a tabulated form of the common aetiological factors of RPL (including REPL) is given in Table 1.

Genetic anomalies have always remained the silent moderators of physiology (Madhual et al., 2023; Kulkarni

et al., 2023). Even before the discovery of modern-day genetic approaches linking disease, people used to identify such diseases as "Inborn Errors of Metabolism" (Haldane, 1954; Harris, 1996). Structural aberrations of chromosomes, like deletion, inversion and translocation, are reportedly associated with several diseases, including some forms of cancers (Queremel et al., 2022; Kloosterman et al., 2014; Albertson et al., 2003). However, in addition to these, alterations in heterochromatin content (h+ or h-) and / or satellite region content (ps+ or ps-) are also being reported by many studies to have certain or uncertain clinical significance (Ferguson et al., 2013; Hong et al., 2011; Belangero et al., 2009). It has already been reported from miscarriage specimen studies that 50 – 70% of miscarriage specimens (Product of Conception or PoC) are due to genetic abnormalities (Levy et al., 2014; Romero et al., 2015; Soler et al., 2017). In fact, miscarriage at the embryonic stage or EPL has the highest prevalence of such anomalies (Romero et al., 2015).

The present study tried to focus on the prevalence of genetic as well as cytogenetic variations among parents or couples with history of REPL or with more than one neonatal death. Diagnosing cytogenetic anomalies was primarily done through karyotyping. Along with this, whole exome sequence analyses were also done based on situational requirements. When available, products of conception (PoC) were also subjected to Chromosomal microarray analyses as part of genetic investigation.

Methods

Subjects and Study Design

A total of 3612 couples experiencing RPL, registered to Mukherjee Fertility Centre, West Bengal, India, from November, 2019 to November, 2023, were the subjects of this study. All were screened for associated clinical parameters and karyotyping studies. A number of them were also subjected to Whole Exome Sequence (WES) analyses as and when required. The males aged between 27 and 46 years (mean age was 35 years) and females aged between 23 and 37 years (mean age 31 years).

Blood collection

A volume of 3 ml peripheral blood was taken from all individuals in vacutainer tubes containing EDTA and mixed up slowly and kept at 4°C until further use.

Leukocyte Culture and Karyotyping:

Leukocyte culture and karyotyping were done following methods described elsewhere (Gerseon et al., 2013). A volume of 0.5 ml whole blood was added to a sterile culture vial containing 5 ml of RPMI-1640 culture medium supplemented with 4 mM of L-Glutamine, 0.1mM gentamycin, 10% foetal bovine serum and phytohemagglutinin or PHA (1.5% final concentration). Then, the contents were mixed gently and incubated at 37°C for 72 hours in the CO2 incubator, maintaining 5% CO2 level, followed by the addition of 20 µL of 50 µg/ml colchicine to arrest mitosis 1 h before culture termination. Harvesting of the peripheral blood leukocytes was performed by treatment with hypotonic solution of potassium chloride followed by fixation in methanol-acetic acid (3:1) and then G-banding was done (350 to 500 band level) (Gerseon et al., 2013). At least 25 metaphases were analysed for each sample and evaluation was increased to 30 metaphases when any abnormality was found in the karyotype. All metaphase plates were evaluated by multiple cytogenetic experts blindedly. Karyotypes were reported according to the International System for Human Cytogenetic Nomenclature (ISCN, 2009; Shaffer et al., 2009).

DNA isolation

DNA isolation was done from peripheral venous blood using "HiPurA SPP Blood DNA Isolation Kit" of Himedia, India, following the manufacturer's instructions strictly. DNA purity check was spectrophotometrically done by checking 260nm / 280nm OD ratio. DNA samples having OD values ranging from 1.8 to 1.9 were only considered for further processing.

Whole Exome Sequence (WES) analyses

WES analyses were done through the hiring service. Sequencing of the protein-coding regions approximately 30Mb of the human exome (targeting approximately 99% of regions in CCDS and RefSeq) was performed using Illumina next-generation sequencing (NGS) systems (NOVASEQ 6000) at a mean depth of 80-100X with percentage of bases covered at 20X depth >90% in the target region.

Chromosomal Microarray analyses

Chromosomal microarray analysis (CMA) was performed using Affymetrix microarray technology supported by their Chromosome Analysis Suite (ChAS) software following the American College of Medical Genetics (ACMG) guidelines 2013 (South et al., 2013). ChAS analysis was based on GRCh37 genome version and Database of Genomic Variations (DGV) database. Analyses will be based on 750,000 copy number analysis markers comprising 550,000 unique non-polymorphic probes and approximately 200,000 single nucleotide polymorphisms (SNPs) covering the genotype with greater than 99% accuracy. Cut-off filters for clinically

DOI: https://doi.org/10.52756/ijerr.2024.v37spl.007

relevant gain/loss and Loss of Heterozygosity (LOH) were 100 kbp and greater than 5 MB, respectively.

Bioinformatic Analyses

To analyse plausible damaging effects of the SNVs being reported here, respective scores and status at Polymorphism Phenotyping v2 (Polyphen-2) (Adzhubei et al., 2010); Sorting Intolerant from Tolerant (SIFT) (Ng et al., 2001); Combined Annotation Dependent Depletion (CADD) (Schubach et al., 2024) and Mutation Taster (MT) (Steinhaus et al., 2021) were used.

Ethics approval and consent to participate

The present study is an observational genetic association study conducted for 3 years to identify possible chromosomal anomalies among couples suffering from idiopathic RPL. This present study was approved by the Institutional Research Board of Mukherjee Fertility Centre, West Bengal, India (Approval No: MFC/IRB/2019-02) strictly based on the Helsinki Declaration of 1975, revised in 2013.

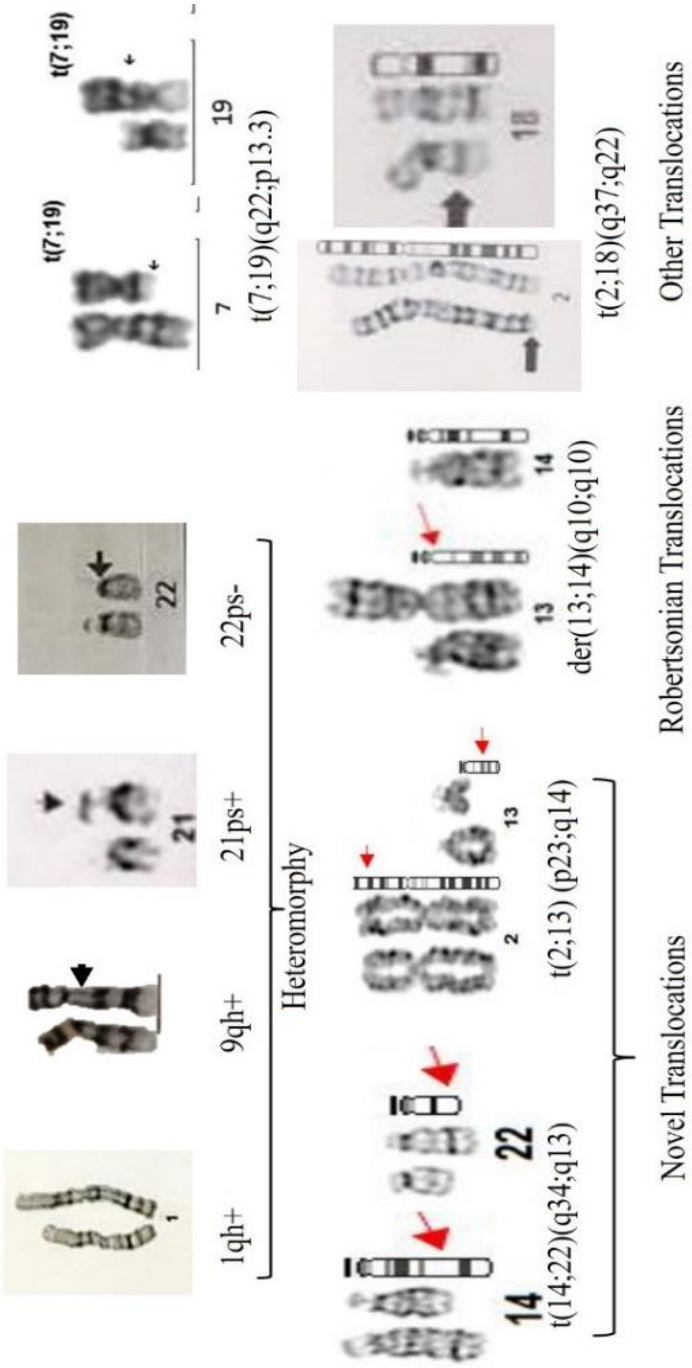
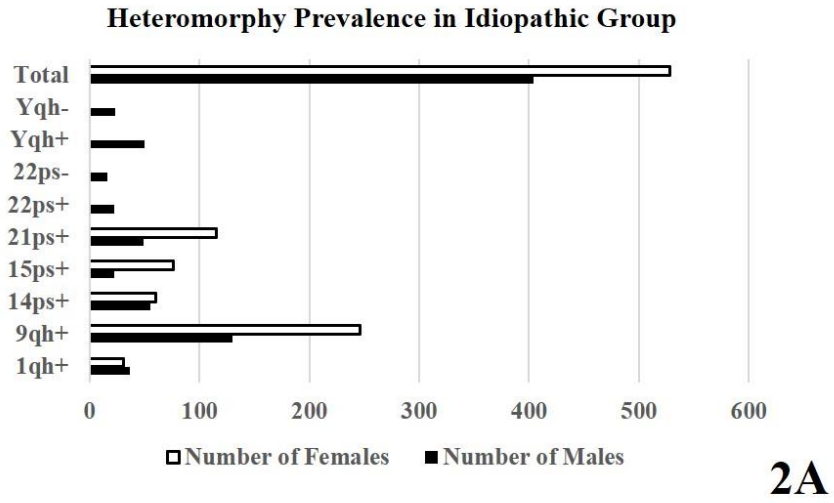
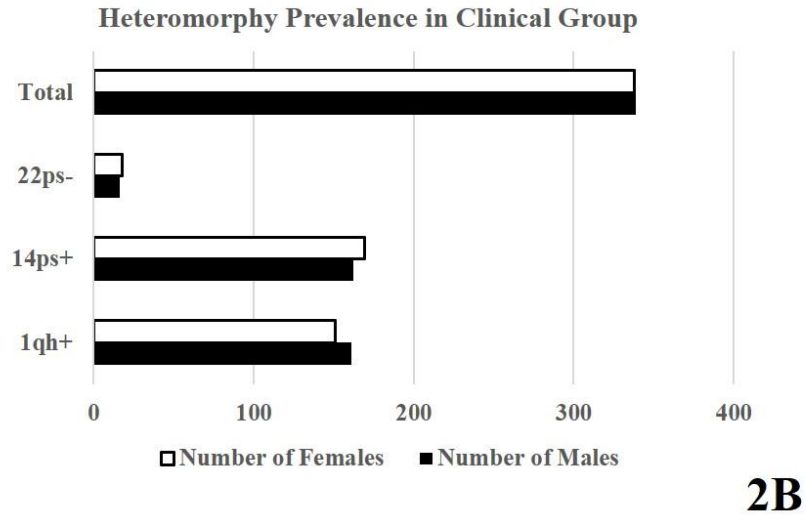


Figure 1. Representative collection of chromosomal anomalies, including heteromorphies and translocations





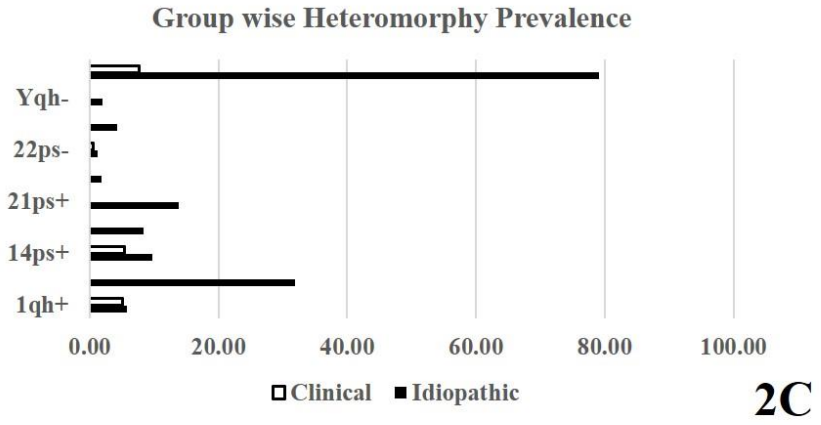


Figure 2. Prevalence of sex specific distribution of chromosomal heteromorphies in idiopathic group (2A), in clinical group (2B) and comparative prevalence percentage of all heteromorphies among idiopathic and clinical group (2C).

Statistical Analyses

Statistical analyses were done using RStudio Statistical package as per requirement.

Results

Among the 3612 couples in the study, 598 were diagnosed as "idiopathic" cases of RPL with a tolerant range of clinical factors summarized in table 1 and

DOI: https://doi.org/10.52756/ijerr.2024.v37spl.007

marked as idiopathic group. The other 3014 couples were marked as "clinical" group as they were suffering from known gynecological problems.

Chromosomal Heteromorphy in Idiopathic group

Of these 598 couples, 51 had structural aberrations in one or both partners, and 11 carried SNVs. Rest of the

Table 2. Chromosomal heteromorphy types and chromosomal aberrations were found associated with RPL

Sl No	Broad type of Chromosomal Anomaly	Specific Anomaly type	Karyotype	Number of Males	Number of Females	Total Number
1	Heteromorphy Including Y linked Heteromorphy	1qh+	46,XX, 1qh+ / 46,XY, 1qh+	37	31	68
2		9qh+	46,XX, 9qh+ / 46,XY, 9qh+	130	246	376
3		14ps+	46,XX, 14ps+ / 46,XY, 14ps+	55	60	115
4		15ps+	46,XX, 15ps+ / 46,XY, 15ps+	22	76	98
5		21ps+	46,XX, 21ps+ / 46,XY, 21ps+	49	115	164
6		22ps+	46,XX, 22ps+	22	0	22
7		22ps-	46,XX, 22ps-	16	0	16
8		Yqh+	46,XY,Yqh+	50	0	50
9		Yqh-	46,XY,Yqh-	23	0	23
			Total Heteromorphy	404	528	932
10	Structural Aberrations	Translocation	t(11;22)(q25; q23)	6	7	13
11			t(3;13)	0	2	2
12			t(14;22)(q24; q13)	4	2	6
13			t(8;17)(q21.2;q24)	3	2	5
15			t(2;13)(p23;q14)^{N}	1	0	1
16			t(2;18)(q37;q22)	1	3	4
17			t(2;12)(p23;q13)	1	1	2
18			t(4;22)(q34;q13)^{N}	3	0	3
			t(6;18)(p21.1;q21.3)^{N}	0	1	1
			t(6;9)(q15;q32)^{N}	1	0	1
19			t(7;19)(q22;p13.3)	2	1	3
20		Inversion	inv(9) (p12;q13)	0	4	4
21			inv(9) (p11;q13)	0	4	4
22			inv(14)(q11.2q13)	2	2	4
23			inv(Y) (p11.2;q11.23)	1	0	1
24			inv(Y) (p11q13)	1	0	1
25		Deletion	delX(q22)	0	1	1
26		Robertsonian Translocation	t(13;15) (q10;q10)	0	1	1
27			t(13;14) (q10; q10)	0	1	1
28	Numerical Aberration	Mosaicism	*46,XX / 45,XX t(p21;p21) (1.1;1.1)	0	1	1
29			46, XY / 47, XXY	3	0	3

DOI: https://doi.org/10.52756/ijerr.2024.v37spl.007

Table 3. Single Nucleotide Variations found in Product of Conceptions (PoCs)- Parental segregation was confirmed by Targeted Sanger Sequencing (dideoxy Chain Termination Method). # Number of applied programs predicting the effect of the variant on the protein outcome: Polyphen-2 Score (P2S1= HumDiv Score, P2S2= HumVar Score); SIFT Details (SIFT=NT means SIFT results Deleterious/Not tolerated); Combined Annotation Dependent Depletion (CADD); Mutation Taster= (MT)

Gene	Variation found among Foetus / Product of Conception	Functional Status	Mode of Transmission	Damage predicting In-silico Parameters#
GCDH	c.1169G>T p.Gly390Val Exon11	VOUS	Homozygous Autosomal Recessive	P2S1=1.000 P2S2=1.000 SIFT= NT CADD= 28.3 MT= Damaging
GCDH	c.1082+1G>A Exon 10	Likely Pathogenic	Compound Heterozygous	-
	c.395G>A p.Arg132Gln Exon 6	Likely Pathogenic		-
BCKDHB	c.1114G>T p.Glu372* Exon 10	Pathogenic	Allelic Homozygous Autosomal Recessive	-
PLEC	c.5540G>A p.Arg1847Gln Exon 31	VOUS	Compound Heterozygous	P2S1=0.999 P2S2= 0.982 CADD= 24.9 MT= Damaging
	c.12341C>T p.Thr4114Met Exon 32	VOUS		P2S1= 1.000 P2S2= 0.999 CADD= 28 MT= Damaging
LAMB3	c.2557-2A>C Exon 17	Likely Pathogenic	Homozygous Autosomal Recessive	-
GAA	c.1358_1361del p.Gly453AlafsTer23 Exon 9	Pathogenic	Homozygous Autosomal Recessive	-
UBE3B	c.1109_1110del p.Val370GlyfsTer5 Exon 12	Pathogenic	Homozygous Autosomal Recessive	-
FTKN	c.411C>A p.Cys137* Exon 5	Pathogenic	Homozygous Autosomal Recessive	-
LAMC2	c.3385C>T p.Arg1129* Exon 23	Pathogenic	Homozygous Autosomal Recessive	-
DYNC2H1	c.9825_9826del p.Cys3277ProfsTer31 Exon 64	Pathogenic	Compound Heterozygous	-
	c.7735C>T p.Arg2579Trp Exon 48	VOUS		P2S1=0.542 P2S2= 0.342 SIFT = **0.02** CADD = 23
TBCK	c.(2059+1_20611) _(2234+1_2236-1)del Exon 23	Likely Pathogenic	Homozygous Autosomal Recessive	-

DOI: https://doi.org/10.52756/ijerr.2024.v37spl.007

couples of were found to carry chromosomal heteromorphies. Along these, 18 males and 37 females were found to carry both heteromorphisms as well as structural aberrations. A representative assemblage of some of the reported anomalies is given in Figure 1.

Heteromorphy percentage was significantly higher in females (86.8%) than males (67.6%) among the idiopathic group. Twentynine (29%) females in the study were found to carry multiple anomalies, significantly higher than their male counterparts (18%). 9qh+ was the highest associated heteromorphy with RPL followed by 21ps+ (Figure 2A).

Chromosomal Heteromorphy in Clinical Group

Presence of chromosomal heteromorphy was also there among the clinical group however, 9qh+, 15ps+ and 21ps+ were absolutely absent. 1qh+ and 14ps+ were found to be the prevalent heteromorphies. Interestingly number of females was again significantly higher than males in carrying heteromorphies (Figure 2B). A comparative prevalence percentage of all heteromorphies is summarized in Figure 2C.

Structural Aberrations of Chromosomes

The present study is reporting association of four novel translocations, viz., 46, XY, t(2;13)(p23; q14); 46, XY, t(4; 22)(q34; q13) 46, XY, t(6;9)(q15; q32) and 46, XX, t(6;18)(p21.1; q21.3) among the ten translocations being reported. Translocation prevalence is comparatively higher in male subjects. Inversions and deletions were also found among the subjects; however, chromosomal duplications were really scanty in our studies (Table 2). Translocations involving Chromosome 22 are found to be the highest, while inversion is prevalently associated with Chromosome 9.

[N] Novel translocations. * This female had 5 events of RPL, fortunately, karyotype report of the last product of conception (PoC) was done, which shows 45,XX, +21, t(p21;p13) (1.1;1.1). # the differences are significant at *P 0.05*.

Prevalence of 1qh+ and 14ps+ were similar among idiopathic and clinical groups, and there was no sex biasness. However, the prevalence and sex distribution of 22ps- remain insignificant in the present study.

Single Nucleotide Variations

PoC materials from eleven idiopathic group couples were found to have homozygous or compound heterozygous variations in the same genes. Parental segregation analyses revealed each of the paternal and maternal partner to be heterozygous for either the same SNV or a different SNV in the same gene (Table 3).

To, the best of the knowledge of the subjects, all of them were non-consanguineous up to their previous four generations on both their paternal and maternal sides. But, even with such non-consanguinity, the same alleles were present among the couples, which was truly a scientifically explorable fact. However, the present study did not have any scope of documenting the subjects' caste, religion, or ethnicity.

Discussion

The present study finds a prevalence of chromosomal anomalies at around 13.15% among the study population, which is higher in comparison to other studies (Alibaksh et al., 2020; Ananthapur et al., 2012; Turki et al., 2016; Chakraborty et al., 2021). Pregnancy loss has always remained a case of major concern since the social impact of this is also very important along with health / clinical issues. Situations get worse if the pregnancy loss is recurrent and idiopathic. Psychological devastation is very common among such couples and their family members. In fact, after the detection of structural problems in the karyotype, one partner of one couple deliberately commented as “then I am the rotten apple” to the genetic counsellor of this study. Structural aberrations of chromosomes have remained a major genetic cause of metabolic disorders. Translocation has remained the major aberration type, followed by inversions and deletions. It has already been reported that there exist several translocation hotspots within the human genome, thus, the frequency of translocation is naturally higher than other anomaly types (Silva et al., 2014; Nesta et al., 2021). In case of balanced translocation parental partner(s) remain/remain unaffected with it however, the zygote produced from these chromosome sets faces the dosage issue. Therefore, structural aberrations and their roles in REPL were explainable.

However, association of chromosomal heteromorphisms with REPL is truly enigmatic. The present study finds that more than 31% and 13% of couples have 9qh+ and 21ps+ karyotypes among the “idiopathic” group, respectively. Since, all other above-mentioned common causes of REPL had been nullified in the subjects, therefore chromosomal heteromorphy remains the only possible cause behind the recurrent pregnancy losses among these couples. Reports regarding chromosomal anomalies among couples suffering from RPL are not scanty (summarized in Table 4) from researchers in other populations.

Interestingly, apart from structural anomalies of chromosomes such as, translocation and/or inversions, heteromorphies are also reported as major variations or polymorphisms in those studies. Among the heteromorphies 1qh+, 9qh+, 14ps+, 15ps+ and 21ps+

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were the predominant ones in both sexes. Chromosome Y linked heteromorphies such as Yqh+ and Yqh- both are significantly found among males with a history of REPL. Though some studies (Turki et al., 2016; Chakraborty et al., 2021) report the presence of 16qh+ among the couples of REPL, the present study did not find a single report of 16qh+ in its study population. All studies cumulatively report higher prevalence of chromosomal heteromorphies in females than males, like our finding. Till date such reports may have clinical implications for among clinical pregnancies reported that the blastocyst rate decreases along with the increase in aneuploidies when zygotes carry chromosomal heteromorphisms (Cao et al., 2022). In continuation, may the present authors propose that the presence of altered heterochromatin and/or satellite regions very close to the centromere may have significant interruptions of HP1 and CPC interplay for successful karyokinesis leading to unsuccessful cellular division, which in turn cause foetal mortality at an early stage due to erroneous embryogenesis.

Table 4. Details of existing RPL reports showing similar findings in other populations. Chromosomal anomalies were primarily divided into two groups here – structural aberrations (translocation / inversion / deletion) and heteromorphy (altered heterochromatin / satellite contents)

Sl No	Broad type of Chromosomal Anomaly	Specific Anomaly type	Reference
1	Heteromorphy	9qh+, 1qh+	Alibakshi et. al., 2020
	Structural aberration	Translocations	
2	Heteromorphy	15ps+	Ananthapur et. al., 2012
	Structural aberration	Novel Translocation 46, XX, t(12;13) (q13;q33)	
3	Heteromorphy	13ps+, 16qh+	Turki et. al., 2016
	Structural aberration	Duplications, Translocations, Robertsonian translocation	
4	Heteromorphy	1qh+, 9qh+, 13pstk+, 13ps+, 14ps+, 16qh+	Chakraborty et. al., 2021
	Structural aberration	Translocations	

idiopathic REPL because all those were however, without discovered or proposed aetiology (Alibakshi et al., 2020; Ananthapur et al., 2012; Turki et al., 2016; Chakraborty et al., 2021). Several studies have also reported that heteromorphy prevalence was significantly higher among REPL couples in comparison to others (Brothman et al., 2006; Yakin et al., 2005; Goud et al., 2009).

Karyotypes revealed that all the heteromorphy types reported here along are situated very close to the centromeric region of the concerned chromosomes. Reports are there in the scientific literature that DNA sequence alterations at centromeric regions result from an increase in the risk of nondisjunction (Boronova et al., 2015; Pokale, 2015; Ward, 2000). Chromosome Passenger Complex (CPC) proteins, along with heterochromatin protein 1 (HP1), successfully control karyokinesis to cytokinesis along with disjunctions in anaphase of cell divisions (Carmena et al., 2012; Trivedi et al., 2020; Ruppert et al., 2018; Sahin et al. 2008). Therefore, it can be hypothesized that h+/ps+ and/or h-/ps- interfere with the cell division process involving functions of HP1 and CPC. Sahin et al. (2008) and South et al. (2013) proposed that excess heterochromatin/satellite region near the centromere may interfere with cell division during embryogenesis and lead to foetal death. A recent study on aneuploidies

Conclusion

Chromosomal heteromorphy is becoming a strong correlation factor behind idiopathic REPL cases. Structural aberrations like translocations and inversions are also significant contributors to this as major causes. Thereafter, idiopathic REPL is not that idiopathic but rather genetic in most cases, with molecular pathology yet to be discovered. This present study strongly recommends further molecular investigation on this objective to elucidate the underlying pathology behind chromosomal heteromorphy-mediated REPL.

Competing interests

The authors declare that they have no competing interests.

Acknowledgements

The authors express their sincere gratitude towards the subjects of the study for their consent to participation in the inclusion of their data in the study. The authors also sincerely thank Revered Swami Kamalasthananda, Principal, Ramakrishna Mission Vivekananda Centenary College, for infrastructural support.

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How to cite this Article:

Koutilya Bhattacharjee Suman Pal, Emili Banerjee and Shiuli Mukherjee (2024). Recurrent pregnancy loss associated cytogenetic and genetic anomalies – study from eastern India. *International Journal of Experimental Research and Review*, *37*(Spl.), 85-95.

DOI : https://doi.org/10.52756/ijerr.2024.v37spl.007