

The Role of Thrombophilia Factors Polymorphism and Cytogenetic Analysis in Recurrent Pregnancy Loss (RPL) in Northeastern Iran

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ABSTRACT

Background & Objective: Recurrent pregnancy loss (RPL) is the occurrence of three or more miscarriages before the 20th week of pregnancy. Thrombophilia factors are one of the common causes of RPL.

Materials & Methods: This retrospective study was performed on women with miscarriages. 620 patients' documents with pregnancy loss were investigated. Based on the number of pregnancy loss, the women were divided into a control group with less than three miscarriages (212) and RPL group (180). Cytogenetics analysis and thrombophilia factors polymorphism tests were performed for all patients.

Results: In the analysis, none of the studied polymorphisms (MTHFR 677 C/T /Factor V Leiden /Prothrombin G20210A/ ACE I/D/ PAI-1) showed a significant relationship between Control and RPL groups (P-value > 0.05). Cytogenetic analysis showed 2 numerical and 9 structural abnormalities among both groups. Statistical analysis indicated a significant association between the number of abortions and age (P value= 0.005, r =0.139). We even realized that there was a significant relationship between polymorphism number and recurrent number of miscarriages (P value= 0.018, r = 0.6).

Conclusion: We showed that polymorphisms analysis for thrombophilia factors is a more precious test than cytogenetics analysis (study of the banded pattern of chromosomes during metaphase of the cell cycle). We even indicated that no association was found between thrombophilia polymorphisms in the control and RPL groups. This means that screening for Factor V Leiden, prothrombin G20210A, MTHFR C677T, ACE I/D, and PAI-1 and cytogenetic analysis in patients with a history of RPL is not recommended.

Keywords: Thrombophilia factors, Cytogenetic, Recurrent Pregnancy Loss, Polymorphism, Chromosomal abnormalities



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Introduction

Recurrent pregnancy loss (RPL) is defined as the occurrence of three or more consecutive miscarriages before the 20th week of pregnancy, which affects 5% of pregnant women (1, 2). However, the American Society of Reproductive Medicine (ASRM) has identified two or more miscarriages as RPL (2, 3). Chromosomal abnormalities, immunological disorders, anatomical disorders of the uterus, infection, endocrine disorders, and environmental changes can cause RPL (4, 5). However, about 40% of cases are idiopathic (5). One of the common causes of RPL is thrombophilia factors that predispose to thrombosis.

Thrombophilia may be inherited (40%) or acquired (1, 6). Hereditary thrombophilia acts as a risk factor for recurrent pregnancy loss, infertility, obstetrical complications (1, 7). Hereditary thrombophilia is a coagulation disorder associated with the occurrence of venous thromboembolism (VTE). This disorder is related to Factor V Leiden (FVL) as the most common reason, prothrombin G20210A mutation, deficiency of antithrombin, protein C and protein S, increase of coagulation factors VIII and XI, and hyperhomocysteinemia (8, 9). As the number of genetic risk factors increases, the risk of reproductive

disorders increases (10). The most common genetic disorders are mutations in the gene encoding Methylene tetrahydrofolate reductase (MTHFR), mutations in the prothrombin gene promoter, and Factor V Leiden mutations (5, 11). Mutations in the MTHFR gene affect folate metabolism and enzymatic activity and cause hyperhomocysteinemia (1, 12). Decreased enzymatic activity can limit ovulation, oocyte maturation, and luteolysis. Accumulation of homocysteine also by producing reactive oxygen species increases embryonic fragmentation, and decreases embryo cleavage and blastocyst production (1, 13, 14). Mutations in the FV rs6025 gene (effective in converting prothrombin to thrombin) lead to the development of FVL, which resists inhibition by activated protein C (APC) and increases the risk of thrombosis (1, 4). Fibrinolysis control genes such as plasminogen activator inhibitor-1 (PAI-1), which inhibits plasmin production and fibrin degradation, can also play a role in RPL (4, 15). Studies have shown an association between idiopathic RPL and rs1799752 polymorphism (I/D) in the angiotensin-converting gene due to its role in vascular contraction and regulation of PAI-1 activity (4, 15). Studies have shown that thrombophilia factors and chromosomal abnormalities are closely related to recurrent miscarriage or the birth of a disabled child. One of the diagnostic ways is to use the thrombophilia panel to check thrombophilia factors and cytogenetic analysis

to detect chromosomal abnormalities. Also, two methods have been suggested to reduce the birth of a disabled child in couples who have recurrent miscarriages: 1- if one of the parents is a carrier, perform chromosomal analysis and perform amniocentesis 2- perform amniocentesis in all subsequent pregnancies (16, 17).

Due to the relationship between thrombophilia disorders and increased RPL risk. The aim of this study was to evaluate the frequency of thrombophilia factors and cytogenetic disorders in the RPL group.

Methods

Sample collection

In this cross-sectional study, a total of 620 women with a history of three or more repeated abortions who were referred to the Department of Molecular Pathology and Cytogenetics at Ghaem Hospital, Mashhad, Iran, from 2011 to 2020 were recruited. Based on the number of abortions, patients were divided into control (212) and RPL (180) groups. The control group included patients with less than three miscarriages, with or without live children in previous pregnancies. Additionally, patients with incomplete medical record and/ or underlying diseases were excluded from this study (Figure 1).

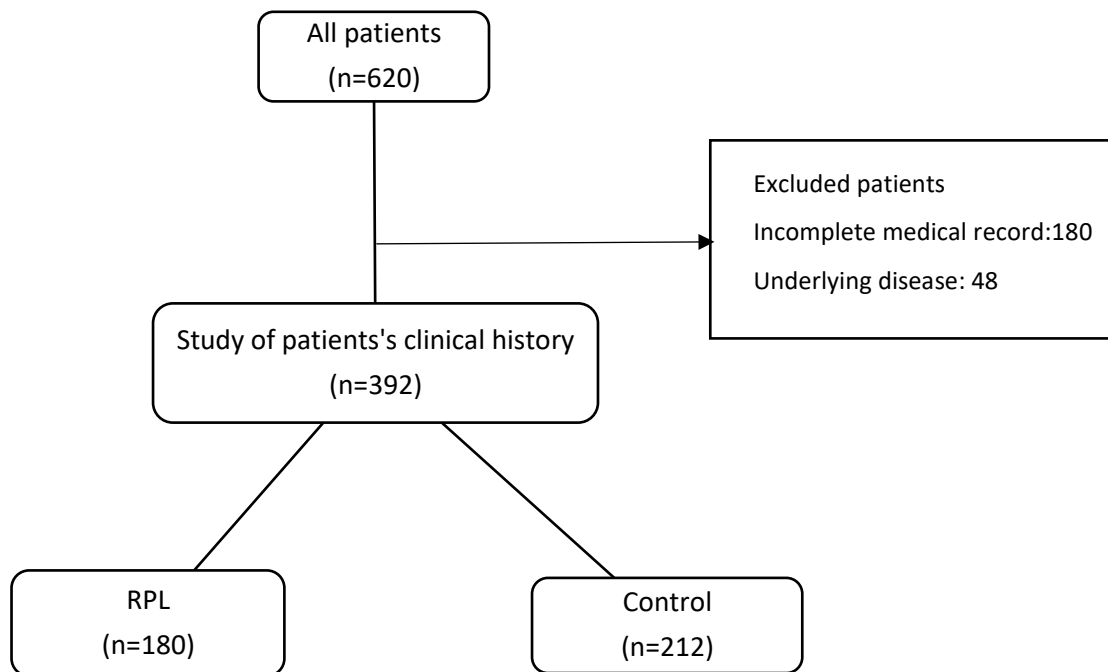


Figure 1. Flow diagram showing patient selection and excluded

Laboratory results and clinical data were collected either from hospital records or from patients' physicians. All patients gave informed voluntary consent agreement to participate in the study, which was approved by the Mashhad University of Medical

Science (MUMS) Ethics Committee. The cytogenetic and thrombophilia panel (MTHFR 677 C/T, FV Leiden G1691A/Prothrombin G20210A (FII), ACEI/D, PAI1) were performed.

Setting up of lymphocyte cultures & karyotyping

Routine cultures of 0.5 ml peripheral blood lymphocytes in RPMI 1640 basal medium with 10% fetal bovine serum (Gibco-Invitrogen-USA) were incubated for 72 hours. Lymphocyte cultures were harvested by addition of colcemid for 10 min (0.075 M), 0.1 microgram/ml of colcemid (Gibco-Invitrogen-USA), and then were treated with hypotonic KCl solution to obtain metaphase. Finally, metaphase chromosomes were spread and stained using the standard G-banding method. In each case, 15 metaphase spreads were studied with Video Test-Karyo software (Version 3.1, Videotest.co, Russia) and at least 50 metaphases were examined where mosaicism was indicated. The International System for Human Cytogenetic Nomenclature (ISCN) recommendations for karyotype reporting were used in 2016 (18, 19).

Genotyping analysis

Genomic DNA was extracted from peripheral blood samples by using the commercially available kit (QIAGEN GmbH, Hilden, Germany). Samples that were used for amplifications had 5 ng/ μ l final concentrations with optical density (OD) 260 nm/280 ratios of 1.5 – 1.8. MTHFR (677 C>T), F2, and F5 thrombophilia gene variants were identified using polymerase chain reaction (PCR) amplification using an improved technique.

The 677 CT substitution in the MTHFR gene was detected using HinfI cleavage of 200bp PCR products, the G1691A polymorphism in factor V Leiden was identified by PCR amplification of a 234 bp fragment and MnlI digestion. For the purpose of identification of the G20210A substitution in the factor II gene, a 345bp fragment from the 3' UTR was amplified by PCR using the same primers as described and digested with HindIII.

Statistical analysis

All results are presented as standard deviation (SD) and mean. Chi square and the independent sample *t*-test, were used for statistical significance of the difference between the two groups. All data analyses

were performed using SPSS software (version 25; IBM SPSS, NY, USA), and a *p* value < 0.05 was considered statistically significant for all variables.

Ethical issues

This study was approved by the ethics committee of Mashhad University of Medical Sciences. All patients' data were obtained from the Cancer Molecular Pathology Research Center of Mashhad University of Medical Sciences. It was one of the articles extracted from the results of project number 931119, under the ethics code of IR.MUMS.REC.1393.963.

Results

In this study, we studied two groups (the RPL group, which includes women with three or more miscarriages and the control group, which includes women with less than three miscarriages with or without live children in previous pregnancies). The mean age was 31.23 \pm 5.15 and 29.51 \pm 5.13 for the RPL and control groups, respectively (Table 1). In this study, we examined the extent of MTHFR, V Leiden, ACE, PAI-1 and Prothrombin G20210A polymorphisms between RPL and the control group. We first examined the prevalence of each polymorphism and its type (Heterozygote, Hemozygote and Wild) between RPL and control groups and reported in Figure 3. Investigation of MTHFR, V Leiden, ACE, PAI-1 and Prothrombin G20210A polymorphisms between RPL and control groups showed that no significant relationship was observed between these polymorphisms and the two groups (P-value>0.05) (Table 1). Cytogenetic analysis has shown that there were 2 numerical and 9 structural abnormalities among 620 patients in our study (Table 2). We also examined the association between the age of individuals with the incidence of pregnancy loss and the association between the number of polymorphisms and pregnancy loss (Figure 2). A significant relationship was observed between the pregnancy loss and the age of individuals in our study (P-value: 0.005, r: 0.139) (Figure 2. A); we even realized that there was a significant relationship between polymorphism number and recurrent number of abortions (P value= 0.018, r= 0.6) (Figure 2. B).

Table 1. Investigation of thrombophilia polymorphisms between Control and RPL groups.

	Control	RPL	P-Value
Age (mean \pm SD)	29.51 \pm 5.13	31.23 \pm 5.15	0.628
MTHFR			
Hetro: N (%)	65(30.7)	50(27.8)	
Homo: N (%)	20(9.4)	14(7.8)	0.635
Wild: N (%)	127(59.9)	116(64.4)	
C20210A			

	Control	RPL	P-Value
Hetro: N (%)	5(2.4)	2(1.1)	0.297
Wild: N (%)	207(97.6)	178(98.9)	
V Leiden			
Hetro: N (%)	2(0.9)	5(2.8)	0.163
Wild: N (%)	210(99.1)	175(97.2)	
PAI1			
Hetro: N (%)	9 (12.7)	9(15)	0.373
Homo: N (%)	3(4.2)	6(10)	
Wild: N (%)	59(83.1)	45(75)	
ACE			
Hetro: N (%)	1(16.7)	0(0)	0.529
Homo: N (%)	2(33.3)	3(42.9)	
Wild: N (%)	3(50)	4(57.1)	
TOTAL			
N (%)	212(100)	180(100)	

Abbreviation: PRL: Recurrent pregnancy loss; Hetro: Heterozygote; Hemo: Hemozygote; MTHFR: methylenetetrahydrofolate reductase; V Leiden: Factor V Leiden; C20210A: Factor II or Prothrombin; ACE I/D: Angiotensin-converting enzyme insertion/deletion; PAI-1: Plasminogen activator inhibitor-1.

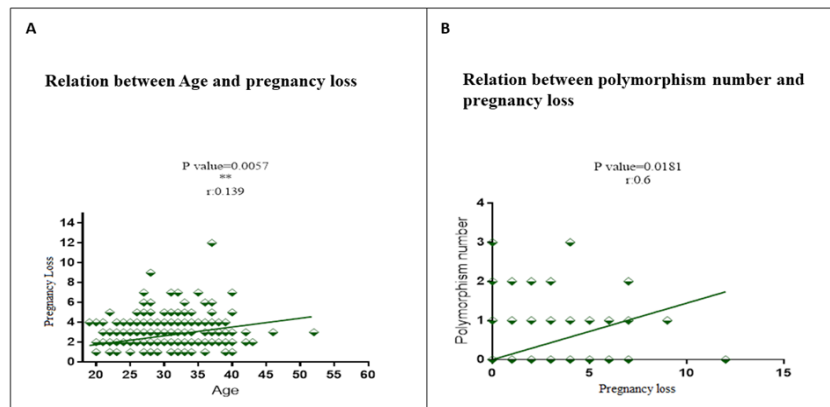


Figure 2. Prevalence of thrombophilia factors: A. Investigating the relationship between the pregnancy loss and the age of the study individuals. B. Investigating the relationship between the pregnancy loss and the polymorphism number.

Table 2. Structural & numerical chromosomal abnormalities identified in this study

Cytogenetic variations	Karyotype	No. of cases
Normal karyotype	46,XX	155
Numerical abnormality	47, XX, +mar[23]/46,XX,[27]	1
Structural abnormalities	46, XX, t(11;22)(q25;q11)	1
	46, XX, t(6;10)(q22.1;q25.1)	1

	45,XX,t(13;21)(q10;q10)	1
	46,XX,inv(X)(p22q26)	1
	46,XX,13pstk+	1
Polymorphic chromosomal variants	46,XX,inv(9)(p12q13)	1
	46,XX,inv(9)(p11q13)	2

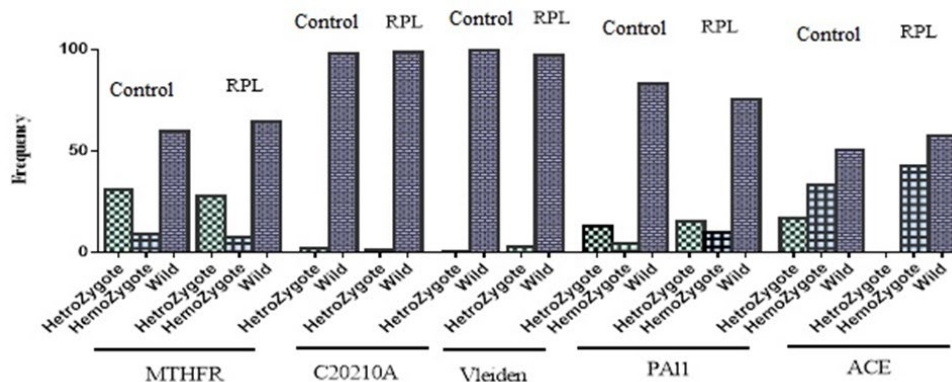


Figure 3. Association between RPL with Polymorphism number. Investigation of the prevalence of each polymorphism and its type separately with each of the RPL and control groups.

Discussion

Thrombophilia panel

In our study, there was no association between the RPL and control groups in terms of thrombophilia factors (MTHFR 677 C/T /Factor V Leiden /Prothrombin G20210A/ ACE I/D/ PAI-1). The rate of MTHFR 677 C/T polymorphism in our study was not significant between the two groups. In another study, Ahangari *et al.* reported that the frequency of MTHFR 677 C/T homozygous mutations was higher in women with RPL than in controls (1). In the study of Yenicesu *et al.*, this ratio was higher in couples with RPL (20). Bigdeli *et al.* showed that MTHFR (677 C/T, 1298 A/C) is associated with RPL (2). The prevalence of homozygous and heterozygous MTHFR 1298 A/C polymorphisms was higher compared to the control group in the study of Behjati *et al.* (21). Zetterberg *et al.* reported a high prevalence of MTHFR 677C/T allele in patients with RPL (22). In contrast, in the study of Dissanayake, there was no association between the MTHFR C677T polymorphism and the RPL (23).

In our study, there was no relationship between FII and FVL between the two groups. In the study of Bigdeli *et al.*, RPL was associated with FV but not with FII (A20210G) (2). In the study of Yenicesu *et al.* among RPL-related couples, the level of homozygous FII was higher only in women than in the control group (20). In the study of Udry *et al.*, the paternal FVL mutation was associated with RPL (24). In the study of

Mohammad *et al.*, the rate of FV was significantly higher in women with RPL (25). In contrast, in the other studies, no correlation was found between FVL and RPL (26-31). In the studies of Ahangari *et al.* and Parand *et al.*, no correlation was observed between FII and RPL (1, 26, 31).

In our study, there was no relationship between ACE I/D and PAI-1 between the two groups. In the study of Bigdeli *et al.*, PAI-1 levels (75675 /I/D, 4G/5G) were associated with RPL (2). In the study of Yenicesu *et al.*, the rate of homozygous mutations of PAI 1 was higher among RPL-related couples in couples higher than the control group, but homozygous ACE I/D was higher only in men associated with RPL (20). In contrast, other Studies showed no association between ACE I/D, PAI-1 4G/5G and increased likelihood of recurrent miscarriage (32-34).

Cytogenetics analysis

In cytogenetic examination between the two groups, there were 2 numerical and 9 structural abnormalities. In our study, a significant relationship was observed between the number of abortions and maternal age. There was also a significant relationship between the number of abortions and the number of polymorphisms.

T. V. Nikitina *et al.* showed in the study between the primary RPL group, in which all pregnancies end by miscarriage, and the secondary RPL group, in which at

least one pregnancy lasts longer than 22 weeks or result in a live birth, the prevalence of abnormal abortion karyotypes in primary RPL was less than is secondary RPL. They also stated that women's age, not obstetric histories, play a major role in the frequency of chromosomal abnormalities in RPL (35).

In one study, Nybo Andersen *et al.* reported Pregnancy loss rates ranged from 8.9% in women under 24 to 74.7% in women 45 of years or more (36).

Awartani *et al.* In a study on couples with RPL showed that the prevalence of chromosomal abnormalities in this group is 7.2% and the prevalence of these abnormalities is higher in women than men. Therefore, cytogenetic evaluation is important in couples with RPL (37). In another study, Alibakhshi *et al.* stated that the rate of chromosomal abnormalities in couples with RPL is 11.5%, so cytogenetic evaluation is recommended in couples with a history of RPL (38).

Limitations and suggestions

In this study, we examined about 620 women with miscarriages; most of the patients were selected from the northeastern region of Iran. Considering the genetic diversity throughout Iran, conducting this study in other regions of Iran can be associated with different and significant results. Therefore, it is recommended to carry out this study in other regions of Iran from a genetic point of view.

In most articles, recurrent miscarriage is interpreted as miscarriage three times or more, and we based our study on the incidence of miscarriage three times or more. However, the American Society of Reproductive Medicine has defined recurrent miscarriage as the occurrence of two or more miscarriages, which can lead to different results.

Conclusion

In our study, there was no association between the RPL and control groups in terms of thrombophilia factors (MTHFR 677 C/T /Factor V Leiden /Prothrombin G20210A/ ACE I/D/ PAI-1), which is probably due to the genetic similarity of the two

groups. Also in this study, the prevalence of thrombophilia factors is higher than cytogenetic disorders, so for RPL screening, the study of thrombophilia factors is preferable to cytogenetic analysis.

Ethical approval

This study was approved by the ethics committee of Mashhad University of Medical Sciences. All patients' data were obtained from the Cancer Molecular Pathology Research Center of Mashhad University of Medical Sciences. It was one of the articles extracted from the results of project number 931119, under the ethics code of IR.MUMS.REC.1393.963

Authors' contributions

N.S has conceived the manuscript and revised it. M.R.J, B.M.B, H.A wrote the manuscript, M.Sh and A.A collected the data, N.A performed the Molecular analysis and E.J, N.S has checked the cytogenetics of patients.

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Data availability

All the data has been included in the manuscript and will be made available upon publication of the manuscript.

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Conflict of Interest

The authors declare no conflict of interest.

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