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RESEARCH ARTICLE

Analysis of polymorphisms in recurrent pregnancy loss: Factor V Leiden G1691A, Factor II G20210A, MTHFR C677T and Factor V H1299R

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ABSTRACT

The distribution of factor V Leiden G1691A, factor II G20210A, MTHFR C677T, and factor V H1299R polymorphisms known to predispose to thrombophilia in 215 cases and 40 controls admitted with indication of recurrent pregnancy loss (RPL) was investigated. Genotyping was performed by melting curve analysis using simultaneous PCR (RT-PCR). There was no difference between genotype and allele frequencies in the case and control groups in terms of the polymorphisms examined ($p>0.05$). In the genotype distribution of the Factor V gene G1691A polymorphism, 12.6% GA in the case group and 90.0% GG (wild) genotypes in the control group were found to be higher than the other genotypes. In the genotype distribution of the factor II gene G20210A polymorphism, the GG (wild) genotype was found to be higher in 94.5% and 97.5%, respectively, in the case group and control group than the other genotypes. In the genotype distribution of MTHFR gene C677T polymorphism, 43.7% CC (wild) in the case group and 40.0% CT genotype in the control group were found to be higher than the other genotypes. In the genotype distribution of the factor V gene A4070G polymorphism, 87.4% AA (wild) in the case group and 80% AA (wild) genotype in the control group were found to be higher than the other genotypes. However, the frequency of risk allele A for factor V Leiden G1691A (6.8% and 5%), the frequency of risk allele A for factor II gene G20210A (3% and 1.2%), MTHFR gene C677T were determined in the case and control groups. The frequency of the T allele (35.9% and 42.5%), which is the risk allele for A4070G, and the frequency of the G allele (6.5% and 16.6%), which is the risk allele for the factor V gene A4070G, were determined. When our study results were evaluated, no relationship was found between RPL and factor V Leiden G1691A, factor II G20210A, MTHFR C677T, and factor V A4070G polymorphisms.

Keywords: recurrent pregnancy loss, factor V, factor II, MTHFR, polymorphism

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INTRODUCTION

Recurrent pregnancy loss (RPL) is defined as the spontaneous termination of at least two or more consecutive pregnancies before the 20th gestational week [1]. Genetic factors play a role in the etiology of recurrent pregnancy loss at a rate of 20%. These factors include chromosomal abnormalities, single gene diseases, mutations in thrombosis susceptibility genes, and multifactorial changes. The incidence of thrombophilia can be seen at a rate of 60% in families with RPL. Thrombophilia is the definition used for conditions that cause hyper coagulation and increase in thrombin formation [2]. Mutations in maternal coagulation factors cause disruptions in the hemostatic system, and placental microcirculation may also lead to thrombosis resulting in abortion. Maternal thrombophilia can cause intrauterine growth retardation and abortions by affecting the circulation and vascular development between the fetus and placenta [3]. Factor V Leiden G1691A, one of the hereditary causes of thrombophilia, is seen in 3-5% of the general population and approximately 20-40% in thromboembolism patients. The prevalence of factor V Leiden

G1691A is between 8-15% in Sweden, lower than 2-4% in Southern Europe and higher in Northern European countries. Its prevalence is reported to be between 5-8% in the United States [4]. Hereditary thrombophilia is most common in the Caucasian population with a prevalence of 10%. Its prevalence in Turkey varies between 3.5-15% [5, 6].

It has been reported that individuals who are heterozygous for the factor II G20210A mutation pose a 2-3-fold risk for thrombosis compared to healthy individuals [7, 8]. Additionally, it has been reported that the risk of abortion increases two to five times in heterozygous women [9].

Disruptions in MTHFR activity prevent the conversion of homocysteine to methionine. Unconverted homocysteine accumulates in the blood plasma and turns the hemostasis balance towards hypercoagulation. Homocysteine accumulating in the vascular fluid can lead to both predisposition to vein thrombosis and RPL during pregnancy, fetal development problems, and premature births [10]. Carrying the CT heterozygous genotype reduces the enzyme activity by 30%, while carrying the TT homozygous mutant genotype reduces the enzyme activity by 70% when compared with the homozygous normal CC genotype in terms of MTHFR C677T polymorphism [11].

In terms of factor V A4070G polymorphism, a relationship has been reported between the GG homozygous mutant genotype and the risk of RPL after 8 weeks of pregnancy [12]. Additionally, factor V A4070G polymorphism AG heterozygous mutant genotype also has a critical role in RPL, and it is recommended to be evaluated as a risk factor [13]. In the current study, the distribution of factor V Leiden G1691A, factor II G20210A, MTHFR C677T, and factor V A4070G polymorphisms known to predispose to thrombophilia in 215 cases and 40 controls admitted with the indication of RPL was examined, and genotyping was performed.

MATERIALS AND METHODS

Determination of Case and Control Groups

In our study, 215 cases out of 1041 women who applied to Afyon Kocatepe University Faculty of Medicine, Department of Medical Genetics outpatient clinic (Afyonkarahisar, Turkey) with the indication of RPL between 2013 and 2018 were evaluated. As the case group, female individuals between the ages of 18-40 who experienced two or more recurrent pregnancy losses before the 20th gestational week were selected. The analysis of factor V Leiden G1691A, factor II G20210A, MTHFR C677T, and factor V A4070G polymorphisms was evaluated retrospectively in the cases.

Female individuals who did not have a chronic or hereditary disease, did not have a history of consanguineous marriage, miscarriage or stillbirth, and had at least two healthy children been considered as the control group. Forty healthy female individuals who met these criteria were included in the study and their ages ranged from 24-55.

Ethics Committee Approval

Ethics committee approval was obtained from Afyonkarahisar Health Sciences University Clinical Research Ethics Committee (with its decision dated 02.08.2019 and numbered 2019/263). Informed consent was obtained from the control group volunteers.

DNA Isolation and RT-PCR Analysis

DNA extraction was performed using the Qiagen EZ1 DNA Blood 200 µL kit from 2 mL peripheral blood samples collected in EDTA tubes from 40 healthy female individuals in the control group. Polymorphism analysis of the controls was studied with the real-time PCR (RT-PCR) method. The DNA samples of the control group were genotyped by performing melting curve analysis on rotor-gene Q (Qiagen) using CVD6 multiplex real-time kit (NML Diagnostic, Italy). However, polymorphism analysis of the case group was performed using the RT-PCR results in the patient files.

Statistical Analysis

Data analysis was performed using the SPSS (statistical package for social sciences) 20.0 (IBM, Chicago, IL, USA) statistical package program. The comparison of the genotype and allele frequencies of the polymorphisms was made using the chi-square (χ^2) test. The difference between the groups compared was considered statistically significant ($p<0.05$).

RESULTS

According to our study results, there was no difference between the case and control groups in terms of factor V Leiden G1691A, factor II G20210A, MTHFR C677T, and factor V A4070G mutations ($p>0.05$).

The genotype distribution for the factor V Leiden G1691A polymorphism was found to be 0.4% (1/215) AA homozygous mutant, 12.6% (27/215) GA heterozygous, and 87.0% (187/215) GG homozygous normal in the case group. In the control group, 0% (0/40) AA homozygous mutant, 10% (4/40) GA heterozygous, and 90.0% (36/40) GG homozygous were normal. When the genotype distribution between the groups was compared, the difference was statistically insignificant ($p=0.935$) (**Table 1**).

There was no difference between the case and control groups in terms of genotypes (AA+GA) carrying at least one copy of the risk allele (A) ($p=0.874$). The frequencies of A and G alleles were 6.8% (29/430) and 93.2% (401/430) in the case group, respectively; in the control group, it was found to be 5% (4/80) and 95% (76/80), respectively. When the allele frequencies were compared between the groups, the statistical difference was insignificant ($p=0.745$) (**Table 1**).

In this study, genotype distribution for factor II gene G20210A polymorphism in the case group was found to be 0.4% (1/215) AA homozygous mutant, 5.1% (11/215) GA

Table 1. Genotype and Allele Frequencies of Factor V Leiden G1691A Polymorphism in Case and Control Groups

Factor V Leiden G1691A	Case	Control	Total	p-value
Genotype frequencies n (%)				
AA homozygous mutant	1 (0.4)	0	1 (0.4)	0.935
GA heterozygous	27 (12.6)	4 (10.0)	31 (12.2)	
GG homozygous normal	187 (87.0)	36 (90.0)	223 (87.4)	
Risk alleles				
GG	187 (87.0)	36 (90.0)	223 (87.4)	0.874
AA+GA	28 (13.0)	4 (10.0)	32 (12.6)	
Allele frequencies n (%)				
A	29 (6.8)	4 (5.0)	33 (6.5)	0.745
G	401 (93.2)	76 (95.0)	477 (93.5)	

Table 2. Genotype and Allele Frequencies of Factor II G20210A Polymorphism in Case and Control Groups

Factor II G20210A	Case	Control	Total	p-value
Genotype frequencies n (%)				
AA homozygous mutant	1 (0.4)	0	1 (0.4)	**0.973**
GA heterozygous	11 (5.1)	1 (2.5)	12 (4.6)	
GG homozygous normal	203 (94.5)	39 (97.5)	242 (95.0)	
Risk alleles				
GG	203 (94.5)	39 (97.5)	242 (95.0)	**0.949**
AA+GA	12 (5.5)	1 (2.5)	13 (5.0)	
Allele frequencies n (%)				
A	13 (3.0)	1 (1.2)	14 (2.8)	**0.833**
G	417 (97.0)	79 (98.8)	496 (97.2)	

Table 3. Genotype and Allele Frequencies of MTHFR C677T Polymorphism in Case and Control Groups

MTHFR C677T	Case	Control	Total	p-value
Genotype frequencies n (%)				
TT homozygous mutant	33 (15.3)	9 (22.5)	42 (16.5)	**0.631**
CT heterozygous	88 (41.0)	16 (40.0)	104 (40.8)	
CC homozygous normal	94 (43.7)	15 (37.5)	109 (42.7)	
Risk alleles				
CC	94 (43.7)	15 (37.5)	109 (42.7)	**0.427**
TT + CT	121 (56.3)	25 (62.5)	146 (57.3)	
Allele frequencies n (%)				
T	154 (35.9)	34 (42.5)	162 (31.8)	**0.436**
C	276 (64.1)	46 (57.5)	348 (68.2)	

heterozygous, and 94.5% (203/215) GG homozygous normal (**Table 2**).

In the control group, the genotype distribution was found to be 0% (0/40) AA homozygous mutant, 2.5% (1/40) GA heterozygous, and 97.5% (39/40) GG homozygous normal. When the genotype distribution between the groups was compared, the difference was statistically insignificant (p=0.973). There was no difference between the case and control groups in terms of genotypes (AA+GA) carrying at least one copy of the risk allele (A) (p=0.949). The frequencies of the A and G alleles were 3.0% (13/430) and 97.0% (417/430) in the case group, respectively. The frequencies of these alleles were found to be 1.2% (1/80) and 98.8% (79/80) in the control group, respectively. When the allele frequencies were compared between the groups, the statistical difference was insignificant (p=0.833) (**Table 2**).

In this study, the genotype distribution for the MTHFR gene C677T polymorphism was found to be 15.3% (33/215) TT homozygous mutant, 41.0% (88/215) CT heterozygous, and 43.7% (94/215) CC homozygous normal in the case group (**Table 3**).

The genotype distribution in the control group was 22.5% (9/40) TT homozygous mutant, 40.0% (16/40) CT heterozygous, and 37.5% (15/40) CC homozygous normal. When the genotype distribution between the groups was compared, the difference was statistically insignificant (p=0.631). There was no difference between the case and control groups in terms of genotypes (TT+CT) carrying at least one copy of the risk allele (T) (p=0.427). The frequencies of the T and C alleles were 35.9% (154/430) and 64.1% (276/430) in the case group, respectively. These frequencies were 42.5% (34/80) and 57.5% (46/80) in the control group, respectively. When the allele frequencies were

Table 4. Genotype and Allele Frequencies of Factor V H1299R Polymorphism in Case and Control Groups

Factor V H1299R	Case	Control	Total	p-value
Genotype frequencies n (%)				
GG homozygous mutant	1 (0.4)	0	1 (0.4)	**0.930**
AG heterozygous	26 (12.2)	8 (20.0)	34 (13.3)	
AA homozygous normal	188 (87.4)	32 (80.0)	220 (86.3)	
Risk alleles				
AA	188 (87.4)	32 (80.0)	220 (86.3)	**0.862**
GG + AG	27 (12.6)	8 (20.0)	35 (13.7)	
Allele frequencies n (%)				
G	28 (6.5)	8 (16.6)	36 (7.0)	**0.735**
A	402(93.5)	72 (83.4)	474 (93.0)	

Table 5. Number of Cases and Rate (%) for Factor V Leiden G1691A Polymorphism in Different Age Ranges

	Genotypes					
	Homozygous mutant		Heterozygous		Homozygous normal (wild type)	
Age ranges	Number of cases	%	Number of cases	%	Number of cases	%
18-24	1	0.4	14	6.5	59	27.5
25-32	0	0.0	10	4.6	85	39.6
33-40	0	0.0	3	1.3	43	20.0

Table 6. Number of Cases and Rate (%) for Factor II G20210A Polymorphism in Different Age Ranges

	Genotypes					
	Homozygous mutant		Heterozygous		Homozygous normal (wild type)	
Age ranges	Number of cases	%	Number of cases	%	Number of cases	%
18-24	0	0.0	5	2.2	69	32.4
25-32	0	0.0	5	2.2	90	42.0
33-40	1	0.4	1	0.4	44	20.4

compared between the groups, the statistical difference was insignificant (p=0.436) (**Table 3**).

The genotype distribution for the factor V gene A4070G polymorphism was found to be 0.4% (1/215) GG homozygous mutant, 12.2% (26/215) AG heterozygous, and 87.4% (188/215) AA homozygous normal in the case group (**Table 4**).

In the control group, the genotype distribution was 0% (0/40) GG homozygous mutant, 20.0% (8/40) AG heterozygous, and 80.0% (32/40) AA homozygous normal. When the genotype distribution between the groups was compared, the difference was statistically insignificant (p=0.930). There was no difference between the case and control groups in terms of genotypes (GG+AG) carrying at least one copy of the risk allele (G) (p=0.862). The frequencies of the G and A alleles were 6.5% (28/430) and 93.5% (402/430) in the case group, respectively. These frequencies were found to be 16.6% (8/80) and 83.4% (72/80) in the control group, respectively. When the allele frequencies were compared between the groups, the statistical difference was insignificant (p=0.735) (**Table 4**).

The number of cases for factor V Leiden G1691A polymorphism was found to be one homozygous mutant (0.4%), 14 heterozygous (6.5%), and 59 homozygous normal (27.5%) in the 18-24 age range; 0 homozygous mutant, 10 heterozygous (4.6%), and 85 homozygous normal (39.6%) in the age range of 25-32, and zero homozygous mutant, three heterozygous (1.4%), and 43 homozygous normal (20%) in the age range of 33-40 years (**Table 5**).

The number of cases for factor II G20210A polymorphism was found to be 0 homozygous mutant, 5 heterozygous (2.2%), and 69 homozygous normal (32.4%) in the 18-24 age range; zero homozygous mutant, five heterozygous (2.2%), and 90 homozygous normal (42%) in the 25-32 age range; and one homozygous mutant (0.4%), two heterozygous (0.9%) and 44 homozygotes normal (20.4%) in the age range of 33-40 years (**Table 6**).

The number of cases in terms of MTHFR C677T polymorphism was found to be 18 homozygous mutants (8.5%), 30 heterozygous (14.2%), and 26 homozygous normal (12.0%) in the 18-24 age group; nine homozygous mutants (4.2%), 44 heterozygous (20.4%), and 42 homozygous normal (19.5%) in the 25-32 age range; and six homozygous mutant (2.7%) in the 33-40 age range; and 14 heterozygous (6.5%) and 26 homozygous normal (12.0%) in the age range of 33-40 years (**Table 7**).

The number of cases in terms of factor V A4070G polymorphism was found to be one homozygous mutant (0.4%), 12 heterozygous (5.6%), and 61 homozygous normal (28.6) in the 18-24 age range; zero homozygous mutant, nine heterozygous (4.2%), and 86 homozygous normal (40%) in the 25-32 age range; and zero homozygous mutant, five

Table 7. Number of Cases and Rate (%) for MTHFR C677T Polymorphism in Different Age Ranges

	Genotypes					
	Homozygous mutant		Heterozygous		Homozygous normal (wild type)	
Age ranges	Number of cases	%	Number of cases	%	Number of cases	%
18-24	18	8.5	30	14.2	26	12.0
25-32	9	4.2	44	20.4	42	19.5
33-40	6	2.7	14	6.5	26	12.0

Table 8. Number of Cases and Rate (%) for Factor V H1299R Polymorphism in Different Age Ranges

	Genotypes					
	Homozygous mutant		Heterozygous		Homozygous normal (wild type)	
Age ranges	Number of cases	%	Number of cases	%	Number of cases	%
18-24	1	0.4	12	5.6	61	28.6
25-32	0	0.0	9	4.2	86	40.0
33-40	0	0.0	5	2.2	41	19.0

Table 9. Number of Miscarriage Cases and Abortion Rates (%)

Parameter	Number of miscarriage				
	2	3	4	5	6
Number of miscarriage cases	130	75	6	3	1
Abortion rates (%)	60.46	34.88	2.79	1.40	0.47

heterozygous (2.2%), and 41 homozygous normal (%) 19.0) in the 33-40 age range (**Table 8**).

According to the available data, the maximum number of RPL was six and the miscarriage rate was 0.47% (1 individual). Out of a total of 215 cases, 75 (34.88%) individuals who had three abortions, 6 (2.79%) individuals who had four abortions, and three (1.40%) individuals who had five abortions were determined (**Table 9**).

DISCUSSION

In our study, the polymorphisms of factor V Leiden G1691A, factor II G20210A, MTHFR C677T, and factor V A4070G genes reported to predispose to thrombophilia were compared in the RPL case and control groups. Although the homozygous genotype of the risk allele in all polymorphic points was higher in the case group than in the control group, this difference was not statistically significant. Similar to our study, it was found homozygous genotypes of thrombophilia gene mutations to be higher in RPL cases compared to controls, but it was not statistically significant [14]. It was found that the frequency of thrombophilia gene mutations was statistically insignificant between RPL cases and control groups [14]. However, they found that homozygous mutations were higher in RPL cases than in the controls. Similarly, in our study, the frequency of homozygous mutant genotype in terms of polymorphisms of factor V Leiden G1691A, factor II G20210A, MTHFR C677T, and factor V A4070G genes was found to be higher in the case group than in the control group.

It was reported that genotype distribution of Factor V Leiden G1691A polymorphism was 0.49% AA, 10.73% GA, and 88.78% GG [7]. Also, the frequency of A allele was found to be 5.85% in the RPL group (205 cases) and 5.5% in the control group (100 individuals). Similarly, the frequency of A allele which poses a risk in factor V Leiden G1691A genotypes was determined as 6.8% in the RPL group and 5% in the control group in our study. The frequency of the risk allele was found to be higher in the case group than in the control group. However, the difference between the two groups for the risk allele frequency was statistically insignificant, which is consistent with [7].

It was reported that the frequency of Factor II G20210A polymorphism was 0.25% for AA genotype and 3.44% for GA genotype in the Southeastern Anatolia Region [15]. In our study, the genotype distribution of factor II G20210A polymorphism was determined as 0.4% for AA, and 5.1% for GA in the RPL group, and 0 for AA, and 2.5% for GA in the control group. It was shown that factor V Leiden G1691A and factor II G20210A polymorphisms were heterozygous, whereas AA homozygous genotype was not detected in 67 patients with RPL (in fetal loss cases at 20th week and above) and 232 control individuals [16]. These authors determined five (7%) individuals for factor V Leiden G1691A and six (9%) individuals for factor II G20210A in the RPL group, while six (3%) and seven (3%) individuals detected for these polymorphisms in the control group. Similarly, in our study, no homozygous genotype was detected in factor V Leiden G1691A and factor II G20210A polymorphisms in the control group. In the control group, factor V Leiden G1691A and factor II G20210A polymorphism were detected in four (10%) and one (2.5%) individual, respectively. In the case group, factor V Leiden G1691A and factor II G20210A polymorphism were detected in 27 (12.6%) and 11 (5.1%) individuals, respectively. In [16], only the data for factor II G20210A polymorphism in the control group were

compatible with our data. It was reported that frequency of factor II G20210A was higher in case group.

In our study, the allele A frequency for the factor II G20210A polymorphism was 3.0% in the case group and 1.2% in the control group, while the allele G frequency was 97.0% in the case group and 98.8% in the control group. Similar results were reported by [17]. The authors studied six polymorphisms associated with thrombophilia (MTHFR C677T, MTHFR A1298C, factor V Leiden G1691A, factor II G20210A, HPA 1 C12548T and APO B R3500Q) in 145 cases and 101 healthy controls. In this study, factor II G20210A polymorphism allele frequencies were found to be similar between the case and control groups. The frequency of the risk allele A was found to be 2.7% and 1.0% in the case and control groups, respectively. In addition, they determined the frequency of the risk allele G as 97.3% and 99%, respectively. These results were similar to the results of our study. Unlike our results, it was determined that there were no individuals with homozygous genotype in terms of factor V Leiden G1691A and factor II G20210A polymorphisms in the case and control groups [17].

In our study, although homozygous mutant genotype (AA) was detected for 1 individual in the case group for factor V Leiden G1691A and factor II G20210A polymorphisms, it was not detected in the control group. It was shown that the genotype of Factor II G20210A polymorphism was GG in 194 cases and GA in 11 cases in a total of 205 cases [7]. These authors did not detect homozygous mutant AA genotype in the RPL group. However, they detected GG genotype in 95 individuals and GA genotype in five individuals in the control group but did not detect homozygous mutant AA genotype. Contrary to [7], homozygous mutant AA genotype was detected for factor V Leiden G1691A and factor II G20210A case group in our study.

In [18], the relationship between RPL and MTHFR C667T and A1298C polymorphisms was investigated in the individuals of 101 cases and 90 controls. It was reported that the polymorphisms examined did not pose a risk for RPL, and there was no difference between the groups in terms of genotypes and allele frequencies [18]. Similarly, in [19], thre was no relationship between MTHFR C677T polymorphism and RPL in which the researchers evaluated 2660 RPL cases. It was reported no association between RPL and MTHFR C677T polymorphism in India on 104 cases and 120 controls, and in Israel on 108 cases and 82 controls [20, 21]. Similarly, our results provide evidence that there is no relationship between MTHFR polymorphisms and RPL.

It was shown that there was no association between the factor V A4070G (factor V H1299R) polymorphism and RPL [22]. This result was similar to the results of our study. It was reported that the frequency of allele A for the factor V A4070G polymorphism was 97.5% and 95.5% in RPL and control groups, respectively [23]. These authors determined that the allele G frequencies were 2.5% and 4.5% in the case and control groups, respectively. These results were similar to our results. The frequency of allele A was 93.5% in the case group and 83.4% in the control group, while the frequency of G allele was 6.5% in the case group and 16.6% in the control group. In a study conducted in Turkey, it was reported that there may be a relationship between RPL and FV G20210A and FVL polymorphisms [19].

There are still unexplained points in the etiopathogenesis of RPL. Studies have reported conflicting findings regarding the relationship between RPL and polymorphisms thought to cause thrombophilia. When our results and the findings of previous the studies are evaluated together, ethnic differences in the sample groups, the small number of samples, and the evaluation of some specific mutations can be counted as the reasons for the conflicting results. Therefore, the effect of genetic perturbations on unexplained RPL can be revealed by conducting studies in which more genes and their polymorphisms are evaluated together in larger sample groups. However, the guideline used for the follow-up of RPL cases was created with data from populations of different origins and populations with different demographic characteristics. For this reason, there is a strong need for studies on this subject in Turkey. As a result, we believe that our study will contribute to the accurate follow-up of RPL cases in Turkey.

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Data sharing statement: Data supporting the findings and conclusions are available upon request from the corresponding author.

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