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Thrombophilia and Recurrent Pregnancy Loss: The Enigma Continues

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Background: Thrombophilic gene polymorphism is known to be a risk factor for recurrent pregnancy loss (RPL), but few studies have confirmed a possible role of thrombophilic genes polymorphism in RPL risk. This study was conducted to understand the relationship of the mutations of some thrombophilia-associated gene polymorphism (heterozygous/homozygous) with RPL. We compared patients with 2 abortions to patients with 3 or more abortions among Turkish women.

Material/Methods: In this study, patients previously diagnosed with habitual abortus at Obstetrics and Gynecology outpatient clinics in Turkey between 2012 and 2016 were included. In their peripheral blood, we detected factor V Leiden H1299R, prothrombin G20210A, MTHFR C677T, MTHFR A1298C, PAI-1 4G/5G, and PAI-1 4G/4G gene mutations.

Results: In this study, we have observed statistically meaningful data ($P<0.01$) related to the relationship between RPL and thrombophilia-associated gene polymorphisms such as heterozygous factor V Leiden H1299R, heterozygous prothrombin G20210A, PAI-1 4G/5G, and PAI-1 4G/4G.

Conclusions: We found that diagnosis of thrombophilic genes polymorphism is useful to determine the causes of RPL, recognizing that this multifactorial disease can also be influenced by various acquired factors, including reproduction-associated risk factors and prolonged immobilization.

MeSH Keywords: Abortion, Habitual • Polymorphism, Genetic • Thrombophilia

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Background

Recurrent pregnancy loss is a real disappointment for married couples. Unfortunately, in many cases the exact underlying pathogenesis of RPL remains undetermined [1].

At the present time, the Practice Committee of the American Society for Reproductive Medicine proposes 2 or more miscarriages as the RPL definition [2], but the European Society of Human Reproduction and Embryology proposes 3 or more miscarriages as the RPL definition [3].

Due to the intensive psychological burden on couples with pregnancy loss, RPL are the most challenging and exhausting areas of reproductive medicine. Because the etiology is not fully known, management-related diagnosis and treatment strategies are limited. Suggested etiologies include chromosomal and uterine anomalies, endometrial infections, endocrine anomalies, antiphospholipid syndrome, hereditary thrombophilia, alloimmune reasons, genetic factors, exposure to environmental factors, and stress factors. Therefore, it is emphasized that a proper examination and standardization be required for RPL patients in order to evaluate potential risks and suitable treatment approaches [4]. The mutation caused by a location change of a single base pair increases prothrombin levels, which in turn raises the risk for thromboembolism. There are 2 types of polymorphism identified due to low efficiency of the MTHFR enzyme. In homozygous individuals, the efficiency level decreases by 35% from normal. Although not at the same level, the efficiency of the enzyme is also reduced in heterozygote individuals. Therefore, the homocysteine level increases [5].

In some other women who have vascular placental pathologies, thrombophilia risk factors are common. For example, in pregnant women with retarded intrauterine development, such as in preeclampsia, late fetal loss and abruptio placenta can be observed [6].

Grees et al. claim that "a disrupted plasminogen" might lead to RPL that stems from proteolysis in women. They suggest that it increases fiber accumulation in early placental circulation and limits the development of trophoblasts, which, in turn, results in RPL. "Hypofibrinolysis" might also cause preeclampsia, fetal growth restriction (FGR), placental abruption, and stillbirth [7,8]. It has been reported that, during trophoblast invasion, urokinase plasminogen activator receptor and plasminogen activator inhibitor-1 (PAI-1) control proteolysis of structures and remodelling of maternal tissues [9].

At present, there is interest in meta-analysis research on inherited thrombophilia, such as studies on the association between thrombophilic gene polymorphism and recurrent pregnancy loss [10], the use of low-molecular-weight heparin inherited thrombophilia [11], association of prothrombin G20210A mutation with recurrent pregnancy loss [12], factor V Leiden mutation in women with early recurrent pregnancy loss [13], and association between plasminogen activator inhibitor-1 (PAI-1) gene polymorphism and recurrent pregnancy loss [14]. Although the findings are inconsistent, many clinicians continue to screen women for thrombophilia because there are reports that combine thrombophilia and reverse pregnancy outcomes. The aim of the present study was to evaluate the etiologies of Turkish RPL patients and to investigate the differences between women with 2 versus 3 or more pregnancy losses.

Material and Methods

Participants

We included patients previously diagnosed with habitual abortus at Obstetrics and Gynecology outpatient clinics in Turkey between 2012 and 2016. We retrospectively analyzed thrombophilia mutation panels of these patients. As case studies, we chose 2660 patients who had at least 2 abortions before the 20th gestational week. In their peripheral blood, we detected factor V Leiden (H1299R), prothrombin G20210A, MTHFR C677T, MTHFR A1298C, PAI-1 4G/5G, and PAI-1 4G/4G gene mutations. We carried out the study after approval was obtained from the Harran University Medical Ethics Committee. Informed consent was obtained from all study subjects.

The eligibility criteria

Patients with 2 or more abortions before the 20th gestational week.

The exclusion criteria

The exclusion criteria were as follows: Patients with self-induced abortion, vaginal infection, systemic diseases, immunological malignancies, uterine structural abnormalities, thyroid diseases, diabetes mellitus, polycystic ovary syndrome or luteal phase defects, environmental factors such as excessive coffee consumption, smoking, alcohol or drug abuse, balanced-type chromosomal translocation, arterial hypertension, liver function abnormalities, and inflammatory pelvic disease.

Molecular diagnosis

For deoxyribonucleic acid (DNA) extraction and molecular analysis, blood samples were collected from each patient into ethylenediaminetetraacetic acid (EDTA) tubes. To determine gene mutations, DNA isolation was carried out from peripheral blood samples. Then, a mutations analysis was conducted with the

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help of strip assay technique, which is based on the reverse hybridization principle. Patients and controls were asked to undergo venous blood collection at least 3 months after pregnancy or cessation of lactation to determine any pregnancy-related alterations of coagulation or fibrinolysis. After a 12-h fast, venous blood was collected in a 1: 10 dilution of 0.11 mol/L trisodium citrate. Blood was centrifuged for 10 min at 2000 g, and serum and plasma were immediately frozen following centrifugation and stored at 80°C. Genomic DNA was isolated from peripheral blood leukocytes with use of a commercially available reagent set (GenXtract Blood DNA Extraction System; ViennaLab Labordiagnostika GmbH).

As described above, we employed PCR and reverse hybridization to genotype samples for factor V Leiden, prothrombin G20210A, methylenetetrahydrofolate reductase (MTHFR) C677T and A1298C, PAI-1 4G/5G, and PAI-1 4G/4G [15].

Genotyping

Genomic DNA was extracted from whole venous blood samples by a commercial DNA isolation kit (Sigma-Aldrich, Taufkirchen, Germany). The MTHFR gene C677T (Ala222Val, rs1801133) and A1298C (Glu429Ala, rs1801131) polymorphisms were examined by polymerase chain reaction (PCR)-based restriction fragment length polymorphism (RFLP) assay. The MTHFR C677T polymorphism was examined by using forward (F) 5'-TGA AGG AGA AGG TGT CTG CGG GA-3' and reverse (R) 5'-AGG ACG GTG CGG TGA GAG TG-3' primers. The amplification conditions included a prior melting phase of 5 min at 94°C; followed by 35 cycles of 30 s at 94°C, 30 s at 61°C, and 30 s at 72°C; and a final elongation phase of 5 min at 72°C. After amplification, the 198 bp PCR product was digested with HinfI. The digestion products were separated on 3% agarose gels, and fragments stained with the ethidium bromide were photographed on an ultraviolet transilluminator. Wild-type (CC) individuals were identified by only a 198 bp fragment, heterozygotes (CT) by both the 175/23 bp and 198 bp, and homozygous variants (TT) by the 175/23 bp. For MTHFR *A1298C* polymorphism, amplification was conducted with primers (F: 5'-CTT TGG GGA GGT GAA GGA CTA C-3' and R: 5'-CAC TTT GTG AGC ATT CCG GTT TG-3'). The amplified 256 bp product was digested with MboII. Wild-type (AA) was determined by 4 fragments (176, 30, 28, and 22 bp), heterozygous AC by 5 fragments (204, 176, 30, 28, and 22 bp), and homozygous variant by 3 fragments (204, 30, and 22 bp). The major visible bands were 204 and 176 bp. A second PCR was carried out to confirm samples whose results were unclear.

DNA fragments were amplified *in vitro*, biotinylated in multiplex PCR reactions, and hybridized for 30 min at 45°C to a membrane test strip presenting a parallel array of allele-specific, 15 to 20 oligonucleotide probes for each mutation: factor V Leiden, 5-GGACAGGC(G/A)AGGAATAC-3; prothrombin, 5-CTCTCAGC(G/A)AGCCTCAA-3; MTHFR C677T, 5-TGCGGGAG(C/T)CGATTTCA-3; MTHFR A1298C, 5-CAGTGAAG(A/C)AAGTGTCT-3; PAI-1 4G/5G, 5-ACACGTGG(/G)GGAGTCAG-3. Specifically-bound PCR fragments were detected with the help of a streptavidin alkaline phosphatase conjugate and color substrates.

Control DNA samples, previously typed by direct DNA sequencing, were available for all mutations and were used to confirm the specificity of the assay. The entire hybridization and detection procedure was performed in a fully automated device (profiBlot II T 30; TECAN, Austria).

Statistical analysis

Data were analyzed using SPSS 22.0 statistical software. The mutation frequencies MTHFR, Prothrombin, FV Leiden, PAI-1 4G/4G, and 4G/5G polymorphisms were examined with the Pearson's chi-square test along with odds ratios with confidence intervals (95%). We performed the *t* test for age, weight, height, and abortus parameters. P-values were reported to be two-sided, and statistical significance was identified to be $P<0.05$. The SAS statistical package, Ver. 8.2 (SAS Institute) was used for all statistical analyses. Depending on the type of data, the Pearson chi-square test or the Fisher exact test (two-tailed) was used. The relative risks for PAI-1 4G/5G were calculated from odds ratios (ORs). Stepwise logistic regression was carried out to determine which genetic thrombophilic factor joined the model as an independent significant risk factor for early pregnancy loss.

Results

We retrospectively analyzed the results of 2660 patients with a history of recurrent abortus who visited our gynecology unit between 2012 and 2016. The average ratio of age, weight, height of the participants, and their number of abortions have been presented in Tables 1–3).

Out of these 2660 patients with a history of recurrent abortus, 1259 were found to have a mutation. The distribution of these mutations is shown in Table 3. The ratios for Factor V Leiden mutation, prothrombin G20210A mutation, MTHFR mutation, and PAI-1 mutation are shown in Table 4.

In the present study, patients with at least 2 abortions were included. As seen in Table 5, when patients with 2 prothrombin G20210A heterozygous abortions and patients with 3 or more abortions were compared, we observed a statistically important difference between the mutation ratio ($P<0.01$). When patients with 2 factor V Leiden H1299R heterozygous abortions and patients with 3 or more abortions were compared,

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Table 1. Socio-demographic distribution (all data included).

Parameter	N	Min.	Max.	Mean	Std. dev. (±)
Age	2660	19	37	26.41	3.517
Weight	2660	58	70	64.96	4.220
Height	2660	150	175	170.82	1.942
Number of abortus	2660	2	6	2.12	.395

Table 2. Socio-demographic distribution analysis of patients with mutations.

Parameter	N	Min.	Max.	Mean	Std. dev. (±)
Age	1259	19	37	26.52	3.54
Weight	1259	58	70	64.86	4.24
Height	1259	150	175	170.76	1.94
Number of abortus	1259	2	6	2.19	.50

Table 3. Analysis of frequency the patients with mutation.

Parameter	Negative		Positive	
	Frequency	Percentage	Frequency	Percentage
f2(Prothrombin) G20210A homozygous	1204	95.6	55	4.4
f2(Prothrombin) G20210A heterozygous	1137	90.3	122	9.7
MTHFR C677T homozygous	1094	86.9	165	13.1
MTHFR C677T heterozygous	1044	82.9	215	17.1
Factor V Leiden H1299R homozygous	1244	98.8	15	1.2
Factor V Leiden H1299R heterozygous	1061	84.3	198	15.7
MTHFR A1298C homozygous	1032	82.0	227	18.0
MTHFR A1298C heterozygous	1043	82.8	216	17.2
PAI-1 4G/5G	1091	86.7	168	13.3
PAI-1 4G/4G	1094	86.9	165	13.1

Table 4. Comparison of demographic data and analysis of the patients with mutation.

Parameter	Results
Age	(19–37) [26.52±3.54]
Weight	(58–70) [64.86±4.24]
Height	(1.50–1.75) [170.76±1.94]
Number of abortus	(2–6) [2.19±.50]
f2(Prothrombin) G20210A homozygous	55/1259 (4.4)
f2(Prothrombin) G20210A heterozygous	122/1259 (9.7)
MTHFR C677T homozygous	165/1259 (13.1)

Parameter	Results
MTHFR C677T heterozygous	215/1259 (17.1)
Factor V Leiden H1299R homozygous	15/1259 (1.2)
Factor V Leiden H1299R heterozygous	198/1259 (15.7)
MTHFR A1298C homozygous	227/1259 (18.0)
MTHFR A1298C heterozygous	216/1259 (17.2)
PAI-1 4G/5G	168/1259 (13.3)
PAI-1 4G/4G	165/1259 (13.1)

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Table 5. Comparison of demographic data and analysis of the patients with mutation between women with two miscarriages and women with three or more miscarriages.

Parameter	Two (n=1695)		Three or more (n=226)		p-Value
Age	(19–37)	[26.36±3.44]	(20–37)	[27.40±3.95]	**0.000**
Weight	(58–70)	[64.81±4.25]	(58–70)	[65.14±4.17]	0.339
Height	(1.69–1.75)	[170.76±1.85]	(1.50–1.75)	[170.74±2.38]	0.911
Number of abortus	(2–2)	[2.00±0.00]	(3–6)	[3.28±0.596]	**0.000**
f2(Prothrombin) G20210A homozygous	43	(4.0%)	12	(6.5%)	0.127
f2(Prothrombin) G20210A heterozygous	87	(8.1%)	35	(18.9%)	**0.000**
MTHFR C677T homozygous	142	(13.2%)	23	(12.4%)	0.769
MTHFR C677T heterozygous	186	(17.3%)	29	(15.7%)	0.583
Factor V Leiden H1299R homozygous	12	(1.1%)	3	(1.6%)	0.473
Factor V Leiden H1299R heterozygous	152	(14.2%)	46	(24.9%)	**0.000**
MTHFR A1298C homozygous	203	(18.9%)	24	(13.0%)	0.503
MTHFR A1298C heterozygous	179	(16.7%)	37	(20.0%)	0.267
PAI-1 4G/5G	123	(11.5%)	45	(24.3%)	**0.000**
PAI-1 4G/4G	121	(11.3%)	44	(23.8%)	**0.000**

we observed a statistically important difference between the mutation ratio (P<0.01). On the other hand, when patients with 2 PAI-1 4G/5G abortions and patients with 3 or more abortions were compared, we observed a statistically important difference between the mutation ratio (P<0.01). However, when patients with 2 PAI-1 4G/4G abortions and patients with 3 or more abortions were compared, we also observed a statistically important difference between the mutation ratio (P<0.01).

Discussion

We examined the relationship between unexplained RPL and thrombophilia gene mutations. In the present study, in terms of weight and height, no meaningful difference was determined between patients with 2 abortions and patients with 3 or more abortions (p>0.05). On the other hand, in terms of the age parameter, we detected a significant difference between patients with 2 abortions and patients with 3 or more abortions (p<0.05).

The causes of RPL are still unexplained in half of the affected patients [16]. For pregnancy complications associated with thrombophilia and hypofibrinolysis, placental deficiencies are common [17,18]. The placenta quickly establishes an arteriovenous anastomosis with endometrium and myometrium, and an excessive thrombosis is formed, which causes placental deficiencies. As a result, complications such as an early abortus, eclampsia, stillbirth, and fetal growth restriction (FGR) can occur. Therefore, it is crucial to make an early diagnosis of thrombophilia and hypofibrinolysis during pregnancy. By doing so, pregnancy complications of women can be eliminated with a low-molecular-weight heparin treatment [19]. In this study, our large-scale clinical trial showed that when patients with 2 Factor V Leiden H1299R heterozygous abortions and patients with 3 or more abortions were compared, we observed a statistically important difference between the mutation ratio (P<0.01). In addition, when patients with 2 PAI-1 4G/5G abortions and patients with 3 or more abortions were compared, we observed a statistically important difference between the mutation ratio (P<0.01). Our literature search found many studies on the relationship between polymorphism gene mutation and pregnancy losses, but these studies have conflicting results [20–22]. For example, in a study by Pıhusch et al., 102 patients with 2 or more abortions and 128 healthy women were compared in terms of F V Leiden, MTHFR C677T, and prothrombin G20210A mutation analyses, showing that there is no difference between the prevalences of MTHFR and F V Leiden. However, for heterozygous prothrombin G20210A mutation, a statistically significant difference was observed between patients with 2 or more abortions and the control group (P=0.027) [23]. Conflicting findings have been reported on the relationship between MTHFR A1298C polymorphism and RPL risk [24,25], but a significant association between MTHFR A1298C polymorphism patients and an increased RPL risk was described [26–28]. For example, in their study titled "MTHFR C677T and A1298C Polymorphism in Iranian Women with Idiopathic Recurrent Pregnancy Losses", Elham et al. did not find a statistically significant relationship

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between MTHFR mutation and RPL [29]. In a similar study by Bae et al., no significant relationship was found between RPL and MTHFR mutation in Korean women [30]. On the other hand, inherited thrombophilia shows different patterns in different ethnic groups, suggesting that among primary and secondary RPL patients, there is no difference between patients with 2 abortions and patients with 3 or more abortions [31]. Although the MTHFR A1298C polymorphism is not responsible for the increase of total homocysteine, this polymorphism has been shown to be a significant contributor to the level of homocysteine [32]. In our study, no significant association between MTHFR C677T polymorphism and RPL was observed in the Turkish population ($p>0.05$). Similarly, no significant association between MTHFR A1298C polymorphism and RPL was observed in the Turkish population ($p>0.05$). Therefore, our findings are contrary to the meta-analysis conducted in China. Previous studies reported that MTHFR C677T polymorphism is significantly related to the risk of recurrent pregnancy losses. In another meta-analysis data set, MTHFR A1298C polymorphism has been shown to be a genetic risk factor for recurrent pregnancy losses [33]. In another study, however, there was no correlation between MTHFR A1298C polymorphism and recurrent pregnancy loss [34]. As for the other 3 polymorphisms, we found a significant association of polymorphisms in prothrombin G20210A, FVL H1299R, and PAI-1 4G/5G with the risk of RPL, which is consistent with the majority of studies [10]. Our findings suggest that, for F2 (prothrombin) G20210A heterozygous mutation, a statistically significant difference was determined between patients with 2 abortions and patients with 3 or more abortions ($p<0.05$). Moreover, we found statistically significant differences between patient groups for factor V Leiden H1299R heterozygous mutation; the patients with 3 or more abortions had a higher ratio than those with 2 abortions ($p<0.05$). For PAI-1 4G/5G mutation and PAI-1 4G/4G mutation variants, there was a statistically significant difference between patient groups. The patients with 3 or more abortions had a higher ratio than those with 2 abortions ($p<0.05$). This finding is in accordance with previous studies. PAI-1 plays a significant intravascular and hemostatic role in arterial and venous thrombosis. After acute arterial injuries and in the event of thrombosis development, PAI-Fx is activated in endothelial cells and rectus [35].

A meta-analysis suggested that future research should focus not only on individual genes, but also on maternal-fetal interaction, gene-gene interactions, genetic-environmental interactions, and other single-nucleotide polymorphisms [36].

Limitations

This was a single-center pilot study that only assessed data from a single private hospital. Multi-center studies with more participants might be useful to advance this area of research.

Strengths

The data analyzed in the present study were obtained from a private hospital, which has a strong network and database.

Conclusions

In this study, we observed statistically significant differences ($P<0.01$) in the relationship between RPL and thrombophilia-associated gene polymorphisms such as heterozygous factor V Leiden H1299R, heterozygous prothrombin G20210A, PAI-1 4G/5G, and PAI-1 4G/4G. Only those patients with additional risk factors like thromboembolism should be considered for a thrombophilia examination. Hereditary thrombophilia demonstrates various patterns in different ethnic groups. The guideline for examination of and care for RPL women relies on data collected from different countries, and data from Turkey are urgently needed. We hope that our study will be of use in counseling RPL women and determining RPL guidelines in Turkey.

Conflict of interest

None.

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