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The association of gene polymorphisms related to inherited thrombophilia with an increased risk of recurrent pregnancy loss

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Abstract

Background Accumulating evidence suggests that inherited thrombophilia may be implicated in the occurrence of recurrent pregnancy loss (RPL) via adversely affecting the placental vascular function. We aimed to investigate the possible association of MTHFR (rs1801133 and rs1801131), FV rs6025, FII rs1799963 and PAI-1 rs1799889 polymorphisms with increased risk of RPL.

Material and methods A total of 320 women with a history of at least two consecutive miscarriages and 100 healthy controls were included in the study. Polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) technique was used for genotyping of the studied single nucleotide polymorphisms (SNPs). Using logistic regression analysis, odds ratio (OR) and 95% confidence interval (95% CI) were calculated to determine the association between the SNPs and RPL, under co-dominant, dominant and recessive inheritance models.

Results The results showed that rs1801133 (OR: 2.158; 95% CI: 1.310-3.553; P = 0.002) was significantly associated with increased risk of RPL, under dominant inheritance model. We also found that rs1799889 was related to a high risk of RPL in co-dominant, dominant and recessive inheritance models. MTHFR rs1801133 T and PAI-1 rs1799889 4G alleles were found to be significantly more prevalent in RPL patients compared to healthy controls (P < 0.05).

Conclusion These data provide strong evidence that rs1801133 and rs1799889 SNPs were significantly associated with increased risk of RPL, suggesting their potential role in RPL pathogenesis.

Introduction

Recurrent pregnancy loss (RPL) is unfortunately one of the most common reproductive health issues, affecting 1-5% of women aiming to conceive worldwide [1]. Currently, cases with two or more consecutive failed clinical pregnancies confirmed by ultrasonography or histopathological examinations are termed as RPL by the American Society for Reproductive Medicine (ASRM) and the European Society of Human Reproduction and Embryology (ESHRE) [2, 3]. RPL is an aetiologically heterogeneous and multifactorial disorder

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involving a combination of multiple genetic and lifestyle/environmental factors [4, 5]. Chromosomal abnormalities, uterine anomalies, metabolic and hormonal disorders, immune dysfunction and genital infections have now been implicated in the etiopathogenesis of RPL [5, 6]. However, the cause of disease still remains shrouded in mystery in almost 50% of cases [5].

Pregnancy is a hypercoagulable state, a physiological strategy that has likely evolved to prevent excessive hemorrhage during and after the delivery [7]. It is therefore unsurprising that women with an increased tendency towards thrombosis due to single nucleotide polymorphisms (SNPs) in one or more genes have a higher risk for RPL. Hyperhomocysteinemia (HHcy), an elevated plasma level of homocysteine (> 15 µmol/L), has been highlighted by previous studies in association with several pregnancy-related complications, especially RPL [8, 9]. It is now documented that HHcy leads to endothelial layer lesions, reducing vessels flexibility and altering haemostasis process, which in turn can induce thrombosis [10]. HHcy may be due to dietary deficiencies of vitamin B6, vitamin B12 and/or folate, and inherited enzymatic defects within the methionine/homocysteine pathway such as C677T (rs1801133) and A1298C (rs1801131) SNPs in the N5, N10-Methylenetetrahydrofolate reductase (MTHFR) gene [11-13]. Coagulation Factor V (FV), also known as labile factor or proaccelerin, plays a dual role in both procoagulant and anticoagulant pathways, acting as a crucial non-enzymatic cofactor [14]. Thereupon, the quantitative imbalance and functional defects of FV can result in either hemorrhage or intravascular thrombosis [15, 16]. The FV G1691A (rs6025) SNP, known as FV Leiden, is the most extensively studied genetic defect leading to hypercoagulability due to poor anticoagulant response to activated protein C (APC) [17]. Prothrombin (coagulation factor II), a multidomain vitamin K-dependent glycoprotein, is the precursor of thrombin, which converts the soluble fibrinogen into insoluble fibrin to form a fibrin network [18]. The FII G20210A (rs1799963) SNP has been found to be associated with elevated levels of plasma prothrombin (hyperprothrombinemia), which in turn lead to hypercoagulability and thrombotic risk [19]. Plasminogen activator inhibitor-1 (PAI-1), also known as SERPINE1, plays a key role in controlling fibrinolysis by inhibiting the tissue plasminogen activator (tPA) and urokinase (uPA) [20]. The PAI-1 -675 4G/5G insertion/deletion (I/D) (rs1799889) SNP directly modulates SERPINE1 gene expression, and the presence of 4G allele enhances transcription to increase plasma PAI-1 levels, which leads to reduced fibrinolysis and increased tendency to thrombosis [21].

To date, numerous studies have investigated the association of rs1801133, rs1801131, rs6025, rs1799963 and rs1799889 SNPs with RPL, but results are conflicting and unconvincing [22- 30]. Accordingly, this study was designed to address the question of whether rs1801133, rs1801131, rs6025, rs1799963 and rs1799889 SNPs can underlie inherited susceptibility to RPL.

Materials and methods

Study participants

In the present case-control study, three hundred and twenty women with two or more consecutive clinically-recognized pregnancy losses and without a history of live births (mean age ± standard deviation (SD); 32.43 ± 5.43 years, range 19 - 47) (mean number of miscarriages ± SD; 2.47 ± 0.92, range 2 - 6) were recruited from Assisted Reproductive Technology (ART) Research Center, Academic Center for Education, Culture and Research (ACECR), Tabriz, Iran, during 2016-2021. To rule out other possible underlying causes of RPL, subjects with chromosomal abnormalities, uterine anomalies, autoimmune diseases, hormonal imbalances and TORCH infections were excluded from the study. The patients' demographic and clinical data, including age, number of miscarriages, preterm births, stillbirths and history of infertility treatment were collected from medical records. Moreover, one hundred ethnicity-matched healthy women with a history of one or more successful pregnancies and no personal and family history of miscarriage (mean age ± SD; 32.75 ± 6.39 years, range 21 - 44) were enrolled as controls. The study protocol was approved by the Institutional Review Board /Independent Ethics Committee (IRB/IEC) of the University of Tabriz, Tabriz, Iran The study was conducted in accordance with the Declaration of Helsinki and written informed consent was obtained from all participants after receiving an explanation of the study.

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DNA extraction and genotyping of studied SNPs

Peripheral blood (~5 mL) was drawn from all subjects into a vacutainer containing ethylenediaminetetraacetic acid (EDTA), and then the genomic DNA was extracted from each sample using the salting-out method described by Miller *et al.* [31]. The quality and quantity of the extracted DNA was determined by measuring the absorbance at 260, 280, and 230 nm using NanoDrop® ND-1000 UV-Vis Spectrophotometer (Thermo Fisher Scientific™, Waltham, MA, USA). We used polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) method for genotyping rs1801133, rs1801131, rs6025, rs1799963 and rs1799889 SNPs. PCR reactions were performed on an Applied Biosystems™ Thermal Cycler (Thermo Fisher Scientific, Waltham, MA, USA) in a final volume of 25 µL containing 12.5 µl Taq DNA Polymerase 2X Master Mix Red (Ampliqon, Denmark), 1 µl of each primer (10 pmol) (Table 1), 100 ng extracted genomic DNA and pure water. The thermal cycling protocol consisted of an initial denaturation at 95 °C for 5 minutes, then 35 cycles of denaturation at 94°C for 40 seconds, annealing at temperature of each primer pair (Table 1) for 40 seconds and extension at 72°C for 40 seconds, followed by a final elongation step at 72°C for 5 minutes. PCR products were electrophoretically separated on 2% agarose gel containing safe DNA gel stain and visualized under ultraviolet (UV) light. To detect rs1801133, rs1801131, rs6025, rs1799963 and rs1799889 SNPs, PCR products were digested by overnight incubation at 37 °C with *HinfI*, *MboII*, *MnlI*, *HindIII* and *BslI* restriction enzymes (New England Biolabs, Beverly, MA, USA) (Table 1), respectively. To identify the genotypes, digested DNA fragments (Table 1) were separated by electrophoresis on polyacrylamide gel stained with silver nitrate.

Statistical analysis

Statistical analysis was performed by IBM SPSS Statistics, Version 22.0 (IBM Corp., Armonk, N.Y., USA) and $P \leq 0.05$ was regarded statistically significant. Departure from Hardy-Weinberg Equilibrium (HWE) for rs1801133, rs1801131, rs6025, rs1799963 and rs1799889 SNPs was calculated using Chi-square test (https://gene-calc.pl/hardy-weinberg-page). We used chi-square test to determine the association between studied SNPs and RPL. Under the co-dominant, dominant and recessive inheritance models, the association of rs1801133, rs1801131, rs6025, rs1799963 and rs1799889 SNPs with risk of RPL was estimated by calculating the odds ratio (OR) and its corresponding 95% confidence interval (95% CI) using logistic regression analysis.

Results

Distribution of genotype and allele frequencies for rs1801133, rs1801131, rs6025, rs1799963 and rs1799889 SNPs in patients with RPL and healthy controls are shown in Table 2. The results indicated that except for rs1799889 SNP ($P < 0.05$), other four SNPs were in accordance with the HWE in the healthy controls ($P > 0.05$) (Table 2). In patients with RPL, the genotype distributions of rs1801133 and rs1799963 SNPs were in agreement with HWE ($P > 0.05$), whereas others were not ($P < 0.05$) (Table 2). We found that the frequencies of rs1801133 and rs1799889 SNPs were significantly higher among RPL patients compared to healthy controls ($P < 0.05$) (Table 2). There were no statistically significant differences between patients with RPL and healthy controls regarding genotype distributions and allele frequencies for rs1801131, rs6025 and rs1799963 SNPs ($P > 0.05$) (Table 2).

Table 1. *List of primers and restriction enzymes used in PCR-RFLP assays for genotyping of the studied thrombophilic gene polymorphisms*

SNP	Primer sequence	Annealing temperature (°C)	Amplicon size (bp)	Restriction enzyme	Fragment length (bp)
rs1801133	5′-TGAAGGAGAAGGTGTCTGCGGGA-3′	61	198	*HinfI*	C allele: 198
	5′-AGGACGGTGCGGTGAGAGTG-3′				T allele: 173, 25
rs1801131	5′-CTTTGGGGAGCTGAAGGACTACTAC-3′	59	163	*MboII*	A allele: 56, 49, 30, 28
	5′-CACTTTGTGACCATTCCGGTTTG-3′				C allele: 84, 49, 30

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rs6025	5′-CATACTACAGTGACGTGGAC-3′	54	206	*MnlI*	G allele: 122, 47, 37
	5′-TGTTCTCTTGAAGGAAATGC-3′				A allele: 159, 47
rs1799963	5′-TCTAGAAACAGTTGCCTGGC-3′	58	345	*HindIII*	G allele: 345
	5′-ATAGCACTGGGAGCATTGAAGC-3′				A allele: 322, 23
rs1799889	5′-CACAGAGAGAGTCTGGCCACGT-3′	60	99, 98	*BslI*	5G allele: 77, 22
	5′-CCAACAGAGGACTCTTGGTCT-3′				4G allele: 98

Abbreviation: PCR-RFLP, polymerase chain reaction-restriction fragment length polymorphism; SNP, single nucleotide polymorphism.

Table 3: *Genotype and allele distribution of studied thrombophilic gene polymorphisms in women with recurrent pregnancy loss and healthy controls*

SNP	**Genotype**	**Cases (n=320) n (%)**	**Controls (n=100) n (%)**	**OR (95% CI)**	***P* value**
rs1801133					0.009
Co-dominant	CC	182 (56.87)	74 (74.00)	1.000	-
	CT	115 (35.94)	22 (22.00)	2.125 (1.250 - 3.611)	0.006
	TT	23 (7.19)	4 (4.00)	2.337 (0.781 - 6.993)	0.172
Dominant	(CC vs. CT+TT)	-	-	2.158 (1.310 - 3.553)	0.002
Recessive	(CC+CT vs. TT)	-	-	1.858 (0.627 - 5.508)	0.351
T allele		21.15	15.00	1.904 (1.242 - 2.919)	0.003
HWE *P* value		0.716	0.389	-	-
rs1801131					0.601
Co-dominant	AA	187 (58.44)	64 (64.00)	1.000	-
	AC	102 (31.87)	27 (27.00)	1.292 (0.776 - 2.154)	0.374
	CC	31 (9.69)	9 (9.00)	1.178 (0.532 - 2.609)	0.703
Dominant	(AA vs. AC+CC)	-	-	1.264 (0.794 - 2.012)	0.351
Recessive	(AA+AC vs. CC)	-	-	1.084 (0.494 - 2.362)	1.000
C allele		25.62	22.50	1.186 (0.814 - 1.729)	0.401
HWE *P* value		0.013	0.078	-	-
rs6025					0.714
Co-dominant	GG	313 (97.82)	99 (99.00)	1.000	-

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	GA	6 (1.87)	1 (1.00)	1.897 (0.225 - 15.95)	0.691
	AA	1 (0.31)	0 (0.00)	ND	ND
Dominant	(GG vs. GA+AA)	-	-	2.214 (0.269 - 18.21)	0.686
Recessive	(GG+GA vs. AA)	-	-	ND	ND
A allele		1.25	0.50	2.519 (0.313 - 20.26)	0.471
HWE *P* value		0.0001	0.998	-	-
rs1799963					0.526
Co-dominant	GG	315 (98.43)	98 (98.00)	1.000	-
	GA	5 (1.57)	2 (2.00)	0.778 (0.148 - 4.072)	1.000
	AA	0 (0.00)	0 (0.00)	ND	ND
Dominant	(GG vs. GA+AA)	-	-	0.778 (0.148 - 4.072)	1.000
Recessive	(GG+GA vs. AA)	-	-	ND	ND
A allele		0.78	1.00	0.779 (0.150 - 4.049)	1.000
HWE *P* value		0.991	0.994	-	-
rs1799889					0.0001
Co-dominant	5G5G	132 (41.25)	74 (74.00)	1.000	-
	5G4G	126 (39.38)	18 (18.00)	3.924 (2.219 - 6.939)	0.0001
	4G4G	62 (19.37)	8 (8.00)	4.344 (1.973 - 9.567)	0.0001
Dominant	(5G5G vs. 5G4G+4G4G)	-	-	4.053 (2.460 - 6.678)	0.0001
Recessive	(5G5G+5G4G vs. 4G4G)	-	-	2.763 (1.274 - 5.991)	0.0083
4G allele		39.06	17.00	3.129 (2.094 - 4.676)	0.0001
HWE *P* value		0.008	0.001	-	-

Abbreviation: OR, odds ratio; CI, confidence interval; HWE, Hardy-Weinberg equilibrium; ND, not determined.

P value were calculated by Chi-square test.

Bold values indicate statistically significant differences ($P < 0.05$).

The association of rs1801133, rs1801131, rs6025, rs1799963 and rs1799889 SNPs with susceptibility to RPL under three inheritance models (codominant, dominant and recessive) were assessed by logistic regression analysis. Under co-dominant inheritance model, CT genotype for rs1801133 SNP revealed a significant association with RPL occurrence (OR: 2.125; 95% CI: 1.250-3.611; $P = 0.006$) (Table 2). The rs1801133 SNP was also found significantly associated with risk of RPL in dominant inheritance model (OR: 2.158; 95% CI: 1.310-3.553; $P = 0.002$) (Table 2). As regards rs1801131, rs6025 and rs1799963 SNPs, no significant association was

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found between genotypic distributions and RPL in any inheritance models ($P > 0.05$) (Table 2). The results showed that rs1799889 SNP was significantly associated with increased risk of RPL under co-dominant, dominant and recessive inheritance models ($P < 0.05$) (Table 2). The allele frequency distribution for studied SNPs was compared between two groups (Table 2). There were no significant differences between RPL patients and healthy controls regarding the allele frequencies of the rs1801131, rs6025 and rs1799963 SNPs ($P > 0.05$) (Table 2). Our findings indicated that the rs1801133 T allele (OR: 1.904; 95% CI: 1.242-2.919; $P = 0.003$) and rs1799889 4G allele (OR: 3.129; 95% CI: 2.094-4.676; $P = 0.0001$) were significantly associated with susceptibility to RPL (Table 2).

Discussion

Inherited thrombophilia has now been accepted as one of the undisputed causes of RPL; however, the association of specific thrombophilic gene polymorphisms with increased susceptibility to recurrent miscarriage still remains controversial [22- 30]. Therefore, we aimed to investigate the possible association between rs1801133, rs1801131, rs6025, rs1799963 and rs1799889 SNPs and occurrence of RPL.

We found a statistically significant association between the rs1801133 SNP and risk of RPL under dominant inheritance model (OR: 2.158; 95% CI: 1.310-3.553; $P = 0.002$). In the co-dominant inheritance model, the rs1801133 CT genotype was significantly associated with RPL susceptibility (OR: 2.125; 95% CI: 1.250-3.611; $P = 0.006$). According to our results, MTHFR rs1801133 T allele can be considered as a genetic risk factor for RPL (OR: 1.904; 95% CI: 1.242-2.919; $P = 0.003$). In line with our findings, Ahangari *et al.* reported that MTHFR rs1801133 SNP had a statistically significant association with RPL risk [30]. In the study conducted by Goodman *et al.* on 550 couples with a history of RPL, they found that MTHFR rs1801133 SNP could predispose the women to RPL [26]. A similar result was obtained in a study (Bigdeli *et al.* 2018) where the association of MTHFR rs1801133 SNP with increased risk of RPL were investigated on 200 RPL patients and 200 healthy women [32]. In 2012, Nair *et al.* reported that MTHFR rs1801133 SNP is associated with occurrence of RPL in the Indian population [33]. A meta-analysis performed by Cao *et al.* in 2013 involving 3559 RPL cases and 5097 healthy controls, demonstrated that MTHFR rs1801133 SNP was significantly associated with RPL susceptibility [34]. These data lead us to conclude that MTHFR rs1801133 SNP which results in reduced enzyme activity and elevated levels of homocysteine can be considered an important risk factor for RPL. However, other studies have not demonstrated any association between this polymorphism and RPL [27, 35, 36]. As regards MTHFR rs1801131 SNP, no statistically significant association was detected under all inheritance models (co-dominant, dominant and recessive) ($P > 0.05$). We also failed to find any significant association between MTHFR rs1801131 C allele and higher risk of RPL occurrence (OR: 1.186; 95% CI: 0.814-1.729; $P = 0.401$). These results reflect those of Yousefian *et al.* who showed that MTHFR rs1801131 SNP is not involved in RPL pathogenesis [35]. Similarly, Poursadegh Zonouzi *et al.* revealed that MTHFR rs1801131 SNP did not have any influence on an individual's susceptibility to RPL [36]. Goodman et al.'s study from the United States showed no significant differences between RPL patients and healthy subjects regarding the genotype distribution and allele frequency of MTHFR rs1801131 SNP [26]. A meta-analysis conducted by Cao *et al.* demonstrated that there is no evidence to support the association of MTHFR rs1801131 SNP with RPL risk [34]. In contrast, two meta-analysis studies have found a statistically significant association between MTHFR rs1801131 and RPL [37, 38]. The results showed that two SNPs, FV rs6025 and FII rs1799963, could not contribute to RPL susceptibility in our population ($P > 0.05$). We found that the prevalence of FV rs6025 A and FII rs1799963 A alleles is extremely low in both groups (≤ 1.25%). Our results are in agreement with previous studies reporting no significant associations between FV rs6025 and FII rs1799963 SNPs with risk of RPL [23, 32, 39, 40, 41]. Conversely, several studies have shown that these SNPs may be related with RPL [42- 49]. In 2002, Finan *et al.* reported that FV rs6025 and FII rs1799963 SNPs are major inherited risk factor associated with RPL [42]. Another study in Egyptian population found a statistically significant association between FV rs6025 and FII rs1799963 SNPs and increased risk of RPL [43]. Previous studies have also demonstrated the relation of FV rs6025 polymorphism, but not FII rs1799963, with RPL [44, 45]. Findings from a meta-analysis showing a significant association between FV rs6025 SNP and RPL susceptibility [46]. Also, two studies suggest that FII rs1799963 SNP, but

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not FV rs6025, is a genetic contributing factor in increasing the risk of RPL [47, 48]. A meta-analysis also revealed a strong association between the FII rs1799963 SNP and RPL [49]. Finally, we found that PAI-1 4G/5G rs1799889 SNP was significantly related to increased susceptibility to RPL under all implanted inheritance models ($P < 0.05$). The PAI-1 4G allele was also detected significantly more frequently in cases than in healthy controls ($P < 0.05$), which is consistent with results obtained in previous studies. Bigdeli *et al.* demonstrated that there was a significant association between PAI-1 4G/5G rs1799889 SNP and RPL risk [32]. Another study conducted in Czech Republic revealed that polymorphism in the PAI-1 gene (rs1799889) can contribute as a risk factor for RPL [50]. When Khosravi *et al.* carried out a case-control study on 421 patients with RPL and 100 healthy subjects, they found PAI-1 4G/5G rs1799889 SNP is associated with susceptibility to RPL [51]. Similar results were also reported by Magdoud *et al.* [52] and Ozdemir *et al.* [53]. A recent meta-analysis also suggested that PAI-1 4G/5G rs1799889 SNP significantly increased RPL risk in Iranian population [54]. However, several studies have failed to find any association between PAI-1 4G/5G rs1799889 SNP and susceptibility to RPL [23, 28, 55]. It is difficult to reach consensus on relation of rs1801133, rs1801131, rs6025, rs1799963 and rs1799889 SNPs with RPL due to inconsistency in the results of studies. Although the reason for these discrepancies is unclear, it can be explained in part by ethnic/racial disparities, differences in sample size and multifactorial nature of disease. These data highlight the need for large-scale studies in diverse populations on different genetic variants to find any conclusive evidence of RPL causes.

Conclusion

In conclusion, our study suggests that MTHFR rs1801133 and PAI-1 4G/5G rs1799889 SNPs may be associated with increased risk of RPL in our population. MTHFR rs1801133 T and PAI-1 4G allele are associated with a significantly increased risk of RPL.

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Conflict of interest

The authors declare that they have no conflict of interest.

Ethical approval

The study protocol was approved by the Institutional Review Board /Independent Ethics Committee (IRB/IEC) of the Tabriz University, Tabriz, Iran.

Informed consent

Written informed consent was obtained from all participants after receiving an explanation of the study.

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