The association between thrombophilic gene mutations and recurrent pregnancy loss

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Abstract

Purpose To determine whether the Factor V (1691G/A), Factor V HR2 (4070A/G), Prothrombin (20210G/A), PAI-1 (-675 I/D, 5G/4G), ACE (intron 16 I/D), Factor VII (Gln353Arg), Factor XIII (Val34Leu), β -fibrinogen (-455G/A), Glycoprotein Ia (807C/T), tPA (intron 8 D/I) gene mutations could be risk factors for recurrent pregnancy loss (RPL).

Capsule It seems that our selected thrombophilic gene mutations is not significantly associated with recurrent pregnancy losses in North western Iranian women.

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Methods Genotyping of thrombophilic gene mutations were carried out by amplification Refractory Mutation System-PCR (ARMS-PCR) method after DNA extraction.

Results We found that the mutant allele frequencies of Factor V (1691G/A), Factor V HR2 (4070A/G), Prothrombin (20210G/A), PAI-1 (-675 I/D, 5G/4G), Factor XIII (Val34Leu) and β -fibrinogen (-455G/A) were more seen in the case group compared with the healthy control; However, the difference between the two group is not statistically significant ($p>0.05$). Whilst the mutant allele frequencies of other studied genes were lower in the case in comparison to the fertile control women ($p>0.05$).

Conclusion Taken together, our data has shown that the prevalence of thrombophilic gene mutations was similar in women with RPL and healthy controls. Therefore, it appears that further studies on large-scale population and other genetic variants will be needed to conclusively find candidate genes for RPL unknown etiology in the future.

Keywords Recurrent pregnancy loss · Thrombophilia · Thrombophilic gene mutations

Introduction

Recurrent Pregnancy Loss (RPL), defined as two or more consecutive pregnancy losses, is a serious reproductive problem, affecting 1–5 % of reproductive-age woman [29, 31]. There is a strong belief that RPL is a multifactorial condition that many factors affect such as chromosomal abnormalities, uterine anatomic malformation, endocrine dysfunction, immunologic factors, infections, and environmental factors [17, 18, 25]. However, the etiology of RPL remains unknown in ~50 % of cases [1].

In an attempt to find candidate genes for RPL, various genetic investigations have been performed on diverse genetic variants. The several studies focused on thrombophilic gene mutations that lead to maternally inherited thrombophilia and their association with RPL [12, 20, 40]. Of note, the possible

role of thrombophilic gene mutations in RPL has been controversial. However, hypercoagulation disorders promoting thrombosis, collectively termed “thrombophilias”, may be inherited or acquired. Although, a part of thrombosis that inherited through genetically, involved nearly 40 % of case [20]. Hereditary thrombophilias resulted from changes in the amount or function of certain proteins that involved in coagulation process [27, 39]. Herein, proposed the pathophysiological roles of maternal thrombophilic gene mutations that able to leading RPL. Additionally, inherited thrombophilias may be lead to blood clots in small vessels in placenta, decreased oxygen delivery to the fetus, and fetal loss [26]. However, the result of several investigations has also been demonstrated an association between thrombophilia disorders and obstetrical complications in early or late pregnancy loss, especially [12, 20, 40].

On this basis, in the current study, in order to address the question of whether maternal thrombophilic gene mutations is due to RPL, we performed examine to analysed the ten thrombophilic gene mutations, that were identified in the most common frequency and related to RPL. The gene mutations included Factor V (1691G/A) [4, 11, 14, 16, 23], Factor V HR2 (4070A/G) [10], Prothrombin (20210G/A) [2, 35], PAI-1 (-675 I/D, 5G/4G) [8, 21], ACE (intron 16 I/D) [8, 21], Factor VII (Gln353Arg) [3, 28], Factor XIII (Val34Leu) [12, 13, 20], β -fibrinogen (-455G/A) [12, 13, 20], Glycoprotein Ia (807C/T) [3, 30] and tPA (intron 8 D/I) [3, 6].

Material and methods

Collection of samples

This investigation was carried out on 89 women with history of at least two consecutive miscarriages, who referred to Women Reproductive Health Research Center (Tabriz, Iran), during 2011–2012. In addition, 50 healthy women with at least two normal pregnancies and no miscarriages were considered as control group. The exclusion criteria for the case group included parents with chromosomal abnormality, uterine anomalies, genital infections, endocrinological disturbances in luteinizing hormone (LH), follicle-stimulating hormone (FSH) and thyroid stimulating hormone (TSH), anti-phospholipid antibodies, and antinuclear antibodies (ANAs). The present study, approved by Ethics and Human Rights Committee and informed consent was obtained from all participants.

Peripheral blood sample (5 ml from each) was taken from all subjects and genomic DNA was extracted from whole blood using salting-out method as previously described [34]. We applied Amplification Refractory Mutation System-PCR (ARMS-PCR) for identification of Factor V, Factor V HR2, Prothrombin, PAI-1, ACE, Factor VII, Factor XIII, β -fibrinogen, Glycoprotein Ia and tPA gene mutations, which are most common reported in the women with RPL. ARMS-

PCR was performed using three primers for each mutation, one forward primer and two reverse primers specific for the wild type and mutant alleles [3]. The PCR amplifications were carried out on total volume 25 μ l solution containing 100 ng genomic DNA, 1 $\times$ PCR buffer, 10 pmol of each primers, 10 nmol each deoxyribonucleotide triphosphates, 1.5 mmol Mg^{2+} and 1 U Taq polymerase. PCR conditions were started with an initial denaturation step (96 $^{\circ}C$, 2 min) was followed by 10 cycles of denaturation (96 $^{\circ}C$, 15 s) and annealing/extension (65 $^{\circ}C$, 60 s), followed by a final 20 cycles of denaturation (96 $^{\circ}C$, 10 s), annealing (61 $^{\circ}C$, 50 s), and extension (72 $^{\circ}C$, 30 s) [3]. The PCR products were separated on 2 % agarose gel and visualized with ethidium bromide.

Statistical analysis

The difference in genotype distributions of each mutation and frequencies of heterozygous, homozygous and mutated alleles among the case and control groups was evaluated using Pearson's chi-square (χ^2 test) and Fisher's exact tests. The homozygote and heterozygote genotypes of each group were unified as a new group and then odds ratios and 95 % confidence intervals were calculated. SPSS software version 16.0 (SPSS Inc., Chicago, IL, USA) was used for statistical analysis and p values <0.05 were regarded statistically significant.

Results

The mean age of women in the case group was 30.18 ± 4.95 years, (ranging between 19 and 42) and the mean age in the control group was 31.54 ± 4.81 years, (ranging between 21 and 44). The mean number of abortions in the case group was 2.94 ± 1.10 (ranging between 2 and 7), and the mean number of successful pregnancy in the control group was 2.2 ± 0.495 (ranging between 2 and 4).

The difference genotype frequencies of the Factor V, Factor V HR2, Prothrombin, PAI-1, ACE, Factor VII, Factor XIII, β -fibrinogen, Glycoprotein Ia and tPA gene mutations in case and control group were shown in Table 1. The rates of genotype frequencies between of the all studied gene mutations, were not different among the both group ($p > 0.05$ χ^2 test) Table 1.

The compared frequencies of the heterozygous mutations of Factor XIII, Glycoprotein Ia, tPA, Factor V, Factor V HR2 and Prothrombin between two group has been showed in Table 2.

The frequencies of mutant alleles for all the studied genes in both case and control subjects were also calculated. Our findings show that the mutant alleles for Factor V, Factor V HR2, Prothrombin, PAI-1, Factor XIII and β -fibrinogen were more frequent in the subjects, while, the frequencies of mutant alleles for ACE, Factor VII, Glycoprotein Ia and tPA were higher in control group (Table 3). The frequencies of total

Table 1 The different genotypic frequencies of the ten thrombophilic gene mutations in case and control groups

Mutation	Case (<i>n</i> =89)			Control (<i>n</i> =50)			<i>P</i> -value ^a
	Normal (%)	Heterozygote (%)	Homozygote (%)	Normal (%)	Heterozygote (%)	Homozygote (%)	
FV 1691G/A ^b	97.75	2.25	0	100	0	0	0.536
FV HR2 4070 A/G ^c	95.51	4.49	0	96	4	0	1.000
F II 20210 G/A ^d	97.75	1.12	1.12	100	0	0	0.566
PAI-1 (-675 I/D, 5G/4G) ^e	29.21	55.05	15.73	30	56	14	0.963
ACE (intron 16 I/D) ^f	25.84	34.83	39.32	14	56	30	0.440
F VII (Gln353Arg) ^g	62.92	33.70	3.37	54	42	4	0.588
F XIII (Val34Leu) ^h	66.29	30.33	3.37	76	22	2	0.483
BF (-455G/A) ⁱ	50.56	42.69	6.74	48	48	4	0.718
G Ia (807C/T) ^j	31.46	56.17	12.35	36	38	26	0.056
tPA (intron 8 D/I) ^k	42.69	29.21	28.08	42	22	36	0.528

FV Factor V; *FV HR2* Factor V-His1299 Arg; *F II* Factor II or Prothrombin; *PAI-1* Plasminogen Activator Inhibitor-1; *ACE* Angiotensin Converting Enzyme; *F VII* Factor VII; *F XIII* Factor XIII; *BF* beta fibrinogen; *G Ia* Glycoprotein Ia; *tPA* tissue Plasminogen Activator

^a Evaluated by Pearson's chi-squared test

^b FV, at nucleotide position 1691 on the gene a G was exchanged by an A

^c FV HR2, at nucleotide position 4070 on the gene an A was exchanged by a G

^d F II, at nucleotide position 20210 on the gene a G was exchanged by an A

^e PAI-1, at nucleotide position -675 on the gene promoter a GGGG was exchanged by a GGGGG

^f ACE, insertion/deletion of an Alu sequence of 287 bp in the intron 16 of the ACE gene

^g F VII, at codon position 353 of the protein was converted a Gln to an Arg

^h F XIII, at codon position 34 of the protein was converted a Val to a Leu

ⁱ BF, at nucleotide position -455 on the gene promoter a G was exchanged by an A

^j G Ia, at nucleotide position 807 on the gene a C was exchanged by a T

^k tPA, Alu-repeat insertion/deletion (I/D) in intron 8 of the tPA gene

Table 2 The frequencies of heterozygote and homozygote genotypes of the ten thrombophilic gene mutations in case and control groups

Mutation	Frequencies of heterozygote genotype (%)			Frequencies of homozygote genotype (%)		
	Case group	Control group	<i>P</i> -value	Case group	Control group	<i>P</i> -value
FV 1691G/A	2.25	0	0.536	0	0	Not estimated
FV HR2 4070 A/G	4.49	4	1.000	0	0	Not estimated
F II 20210 G/A	1.12	0	1.000	1.12	0	1.000
PAI-1 (-675 I/D, 5G/4G)	55.05	56	1.000	15.73	14	1.000
ACE (intron 16 I/D)	34.83	56	0.020	39.32	30	0.357
Factor VII (Gln353Arg)	33.70	42	0.363	3.37	4	1.000
Factor XIII (Val34Leu)	30.33	22	0.327	3.37	2	1.000
BF (-455G/A)	42.69	48	0.596	6.74	4	0.711
G Ia (807C/T)	56.17	38	0.052	12.35	26	0.060
tPA (intron 8 D/I)	29.21	22	0.426	28.80	36	0.346
Total heterozygote genotype ^a	28.98	28.60	1.000	—	—	—
Total homozygote genotype ^b	—	—	—	11.01	11.60	1.000

FV Factor V; *FV HR2* Factor V-His1299 Arg; *F II* Factor II or Prothrombin; *PAI-1* Plasminogen Activator Inhibitor-1; *ACE* Angiotensin Converting Enzyme; *F VII* Factor VII; *F XIII* Factor XIII; *BF* beta fibrinogen; *G Ia* Glycoprotein Ia; *tPA* tissue Plasminogen Activator

^a The frequencies of total heterozygote genotypes for all studied gene mutations

^b The frequencies of total homozygote genotypes for all studied gene mutations

mutant alleles among cases and controls were 25.50 % and 26 % respectively, which showed any meaningful differences between two group ($p=0.786$) (Table 3).

When the heterozygote and homozygote genotypes of each group were unified into a new group (carrier group), and then odds ratios and 95 % confidence intervals were calculated we failed to show an association between all studied gene mutations and RPL (Table 4).

Discussion

We investigated the association between early recurrent fetal loss and maternally thrombophilic gene mutations. This is the first report to study the prevalence of ten thrombophilic gene mutations in North western Iranian women with a history of RPL. We found that the mutant allele frequency for FV, Factor V HR2 and Prothrombin mutations were low prevalence in both group. These results proposed that Factor V, Factor V HR2 and Prothrombin mutations are not widespread in our population and screening for these mutations not recommend in assessment of patients who suffered from RPL. However, our study determined the mutant allele frequency for PAI-1, ACE, Glycoprotein Ia and tPA were more prevalence in both group (Table 3). Therefore, these results might be proposed the PAI-1, ACE, Glycoprotein Ia and tPA mutations are widespread in North western Iranian women and not revealed as a risk factors in pregnancy loss, but could also be considered as heritable variants.

In contrast with some previous reports that revealed a positive association between maternal thrombophilic gene

mutations and RPL [12, 20, 40], we failed to show a relationship between all studied gene mutations and reproductive failure in women who live in the Northwest of Iran. Even the heterozygote and homozygote genotypes of each group that were unified into carrier group and then odds ratios and 95 % confidence intervals were calculated, we could not find an association between thrombophilic gene mutations and RPL (Table 4). This may be a consequence of racial differences populations or using low number of subjects in the present study. In the other hand, many factors affecting in pregnancy, such as age at pregnancy attempt, regularity of the menstrual cycle, appropriate implantation, immunological tolerance of the fetus, social behavioral, and environmental factors.

Factor V and prothrombin

Several studies showed that Factor V and Prothrombin are the two most common studied thrombophilic genes that related to fetal loss. Carp et al. [9] reported that the prevalence of the FV and Prothrombin mutations as (6.1, 6.1) % and (3.7, 4.6) % for controls and patients, respectively. In addition, Ghee and Burrows [19] clarified that there is no evidence to support an association of Prothrombin mutation with RPL. In study by Altintas et al. [2] revealed that the frequency of FV mutation was similar in patients with RPL and controls. Similarly, our findings showed the incidences of FV and Prothrombin mutations were identity in both groups.

Table 3 The frequencies of mutant allele of the ten thrombophilic gene mutations in case and control groups

Mutation	Frequency of mutant allele (%) in case group	Frequency of mutant allele (%) in control group	<i>P</i> value ^a
FV 1691G/A	1.12	0	0.409
FV HR2 4070 A/G	2.24	2	0.628
F II 20210 G/A	1.68	0	0.261
PAI-1 (-675 I/D, 5G/4G)	43.25	42	0.470
ACE (intron 16 I/D)	56.74	58	0.470
Factor VII (Gln353Arg)	20.22	25	0.219
Factor XIII (Val34Leu)	18.53	13	0.153
BF (-455G/A)	28.08	28	0.551
G Ia (807C/T)	40.44	45	0.270
tPA (intron 8 D/I)	42.69	47	0.285
Total mutant allele	25.50	26	0.786

FV Factor V; *FV HR2* Factor V-His1299 Arg; *F II* Factor II or Prothrombin; *PAI-1* Plasminogen Activator Inhibitor-1; *ACE* Angiotensin Converting Enzyme; *F VII* Factor VII; *F XIII* Factor XIII; *BF* beta fibrinogen; *G Ia* Glycoprotein Ia; *tPA* tissue Plasminogen Activator

^a Evaluated by chi-squared test

Factor V HR2

Previously, Castoldi et al. [10] has been shown that the Factor V HR2 mutation associated with mild activated protein c resistance (APCR) and a mild risk factor for thrombosis. However, similar to our findings, in the several of assessments, revealed that not seen statistically significant differences in Factor V HR2 mutation between patients and normal group [12, 20, 40].

ACE and PAI-1

Contradictory of the recent study by Subrt et al. [38] that demonstrated a strong positive association between PAI-1 and ACE mutations and pregnancy loss [38], in our study and other authors, no relationship was founded between the presence of ACE and PAI-1 mutant alleles and RPL [8, 15, 21, 22]

Table 4 Odds ratios (95 % CI) for the ten thrombophilic mutations in case and control groups

Mutation	Case (<i>n</i> =89)		Control (<i>n</i> =50)		<i>P</i> -value	OR	95 % confidence interval of an odds ratio
	Carrier ^a (%)	Non-carrier ^b (%)	Carrier ^a (%)	Non-carrier ^b (%)			
FV 1691G/A	2.25	97.75	0	100	0.536	1.023	0.991–1.056
FV HR2 4070 A/G	4.49	95.51	4	96	1.000	1.129	0.199–6.395
F II 20210 G/A	2.25	97.75	0	100	0.536	1.023	0.991–1.056
PAI-1 (-675 I/D, 5G/4G)	70.79	29.21	70	30	1.000	1.030	0.487–2.216
ACE (intron 16 I/D)	74.16	25.84	86	14	0.133	0.467	0.184–1.183
Factor VII (Gln353Arg)	37.08	62.92	46	54	0.363	0.692	0.342–1.397
Factor XIII (Val34Leu)	33.71	66.29	24	76	0.254	1.610	0.735–3.526
BF (-455G/A)	49.46	50.56	52	48	0.860	0.903	0.451–1.805
G Ia (807C/T)	68.54	31.46	64	36	0.707	1.225	0.590–2.544
tPA (intron 8 D/I)	57.31	42.69	58	42	1.000	0.972	0.482–1.960

FV Factor V; *FV HR2* Factor V-His1299 Arg; *F II* Factor II or Prothrombin; *PAI-1* Plasminogen Activator Inhibitor-1; *ACE* Angiotensin Converting Enzyme; *F VII* Factor VII; *F XIII* Factor XIII; *BF* beta fibrinogen; *G Ia* Glycoprotein Ia; *tPA* tissue Plasminogen Activator

^a Carriers: individuals who had either heterozygous or homozygous specified mutation

^b Non-carriers: individuals who had neither heterozygous nor homozygous specified mutation

Glycoprotein Ia

Several investigations have performed to find coloration between Glycoprotein Ia mutation and thrombotic disorders [7, 30, 32] But, there are no reported this mutation in women with RPL. Here, we assessed the possible association of Glycoprotein Ia mutation and RPL. The results of present study showed that there is no evidence to support an association of Glycoprotein Ia with RPL in our population.

tPA

Previous studies have found positive link between the Alu-repeat insertion/deletion (I/D) mutations within the intron 8 of the tPA gene and thromboembolic disease [5, 33, 37]. Similarly Glycoprotein Ia mutation, there are no published studies that have been reported the prevalence of tPA mutation in women with RPL. We failed to show an association between this mutation and miscarriage. Furthermore, in our population seem that tPA mutation could not be risk factors for pregnancy loss.

FVII

Our findings was concordant with earlier publication [36], which reported no association between FVII mutation and RPL. In addition, our findings showed that the FVII mutation was more frequent in healthy control. Therefore, this result led to the view that present mutation does not involved in pregnancy outcome. However, according to our result, we

suggesting that FVII mutation probably have no significant role in the etiology of first trimester RPL in North western Iranian women.

FXIII

Effects of the FXIII mutation on RPL are conflicting. Our results was concordant to earlier studies [12, 15], which reported no association between FXIII mutation and RPL. In contrast of our results, other studies reported a positive correlation between Factor XIII mutation and RPL [20, 40]. Interestingly, the results of the some authors indicated the frequencies of mutations and heterozygosity for FXIII were significantly increased among patients group [20, 40]. However, we also observed that the heterozygosity for FXIII was prevalent in RPL patients but this difference was not statistically significant.

β-fibrinogen

Humphries et al. [24] has been shown the -455G/A mutation of in the β-fibrinogen gene related with higher plasma fibrinogen levels. Similar with the results of reported by Yenicesu et al. [40], we found no significant difference in the prevalence of β-fibrinogen between two groups in our population. In the other hand, Coulam et al. [12] has been reported that whilst none of the specific thrombophilic gene mutations appear to be a risk factor for recurrent miscarriage on their own, when taken together, the total number of mutations form a significant risk. Thus, it appears that the risk for pregnancy loss

might be related to the accumulation of thrombophilic mutations rather than to a specific mutation in women. Hence, this study suggested screening for thrombophilic gene mutations in the pregnancy, especially in women with personal or family history of venous thrombosis, and/or pregnancy thrombosis-associated complications. The results of present study showed that the accumulation of mutated alleles in an individual may be not correlated with recurrent miscarriage in our population.

However, in spite of the negative correlation reported in this study, possible correlation between maternal thrombophilic gene mutations with RPL could not be completely ruled out and testing for some of these gene mutations seem to be useful in the evaluation of patients with RPL in populations that significant differences has been observed between case and healthy control. However, further studies on larger-scale populations may be needed, whilst to better the understanding the pathobiology of RPL disease, we need to identify novel genetic variants and the interaction of these variants with each other and the environment.

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