

A preliminary study of inherited thrombophilic risk factors in different clinical manifestations of venous thromboembolism in central Iran

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Background & objectives: Inherited thrombophilia is known to be an important risk factor for developing venous thromboembolism. Whether such abnormalities may impact the development of deep vein thrombosis (DVT) and pulmonary embolism (PE) differently is not well defined. This preliminary study was undertaken to compare thrombophilic polymorphism in patients with DVT and PE.

Methods: A total of 35 DVT, 23 DVT/PE, and 37 PE patients admitted to the Hajar Hospital, Shahrekord, Iran, between October 2009 and February 2011 were included in the study and 306 healthy volunteers matched by age and sex from the same geographical area with no history of venous or arterial diseases were included as control group. Factor V Leiden (*FV* 1691G/A, rs6025), prothrombin (*FII* 20210G/A), methylene tetrahydrofolate reductase (*MTHFR* 677C/T, rs1801133), and *PLA2* polymorphisms of platelet glycoprotein IIb/IIIa (*GpIIIa* 1565T/C, rs5918) were investigated by polymerase chain reaction-restriction fragment length polymorphism.

Results: The number of patients with the investigated polymorphisms and homozygous carriers was significantly different among the groups ($P < 0.05$). No significant difference was observed in the presence of *FV* 1691G/A and *FII* 20210G/A between any of the patients groups and the control group. *GpIIIa* 1565T/C and homozygous *MTHFR* 677C/T polymorphisms were higher in DVT patients compared with the control group (OR=6.65, 95% CI=3.09-14.30 and OR=4.08, 95% CI=1.35-12.38, respectively).

Interpretation & conclusions: As none of the investigated polymorphisms were associated with PE, other thrombophilia polymorphisms may have a role in the pathogenesis of PE in these patients and should be investigated. Because of different prognostic risk factors among different types of patients, the treatment approach could be different.

Key words Deep vein thrombosis - *GpIIIa* 1565T/C polymorphism - pulmonary embolism - thrombophilia - thromophilia risk factors - venous thromboembolism

Venous thromboembolism (VTE) remains a serious medical condition in patients with known or unknown risk factors causing significant morbidity and mortality¹. VTE manifests as deep vein thrombosis (DVT) and pulmonary embolism (PE). Thrombophilia is defined as a predisposition to increased risk of VTE. The importance of genetic thrombophilia factors in developing VTE has been increasingly recognized. Inherited gene disorders related to the haemostatic system have been documented as risk factors in different studies²⁻⁵.

Factor V Leiden (*FV* 1691G/A, rs6025), prothrombin gene mutation G20210A (*FII* 20210G/A), the variant 677C/T of methylene tetrahydrofolate reductase (*MTHFR* 677C/T, rs1801133) and polymorphism A2 (*PLA2*) in platelet glycoprotein GPIIb/IIIa (*GpIIIa* 1565T/C, rs5918) are among main inherited risk factors. The prevalence of these polymorphisms is different according to ethnicity in normal population and VTE patients²⁻⁴. Whether these abnormalities may impact differently PE and DVT is not well defined, and the available data are controversial. *FV* 1691G/A is a mutation which makes factor V less susceptible to cleavage by activated protein C, *FII* 20210G/A polymorphism is a variant that leads to increased plasma prothrombin level⁵, 677C/T polymorphism of *MTHFR* leads to an elevation of homocysteine in plasma^{6,7}, and *GpIIIa* 1565T/C polymorphism of *GPIIb/IIIa* increases the affinity of IIb/IIIa receptor to fibrinogen and makes the platelet more susceptible to aggregation⁸. Several studies demonstrated increased incidence of DVT than PE in carriers of *FV* 1691G/A⁹⁻¹¹, but others could not find such increased risk¹². Such different clinical manifestations have not been reported for *FII* 20210G/A⁹⁻¹¹.

As there are limited data to compare PE and DVT in the context of thrombophilia in non-Caucasians, this preliminary study was conducted to compare thrombophilia polymorphisms in patients with PE and DVT and to evaluate whether such abnormalities impact these two types of patients in central Iran differently.

Material & Methods

Patients and study design: The study population comprised 35 DVT, 23 DVT/PE, and 37 PE patients who were admitted to the Hajar hospital, Shahrekord, Iran. The patients consecutively enrolled between October

2009 and February 2011 were included in the study. DVT was diagnosed by compression ultrasonography, Doppler ultrasonography, and D-dimer. PE was diagnosed by clinical presentation, chest X-ray, ventilation perfusion lung scan, electrocardiogram, and laboratory findings (blood gases and D-dimer). Patients entered into the study if they had one or more episodes of PE or DVT. Control group included 306 healthy volunteers (faculty and staff of the same hospital, matched by age and sex) from the same geographical area without history of venous or arterial diseases. Ethical approval for the study was obtained from Shahrekord University of Medical Sciences' Ethics Committee. Written consent was obtained from all patients and control participants.

All patients and control participants underwent screening for investigated selected thrombophilia polymorphisms. Five ml of venous blood was collected from the antecubital vein without venous stasis. Genomic DNA was isolated from fresh blood or frozen samples. Polymerase chain reaction followed by restriction fragment length polymorphism was performed for the four polymorphisms (Thermocycler ACTEC, PC818, Japan). Primers and restriction enzymes (Tagc, Denmark and Fermentas, Russia) are shown in Table I.

The *MnI* restriction enzyme digested the 267bp fragment of *FV* 1691G/A in to two fragments of 200bp

Table I. Restriction enzymes and primer sequences

Genetic polymorphism	Restriction enzyme	Primer sequences
<i>FV</i> 1691G/A	<i>MnI</i>	F 5' TGC CCA GTG CTT AAC AAG ACC A 3' R 5' TGT TAT CAC ACT GGT GCT AA 3'
<i>FII</i> 20210G/A	<i>HindIII</i>	F 5' TCT AGA AAC AGT TGC CTG GC 3' R 5' ATA GCA CTG GGA GCA TTG AAG C 3'
<i>PLA1/A2</i>	<i>MspI</i>	F 5' TTC TGA TTG CTG GAC TTC TCT T 3' R 5' TCT CTC CCC ATG GCA AAG AGT 3'
<i>MTHFR</i> 677C/T	<i>HinI</i>	F 5' TGA AGG AGA AGG TGT CTG CGG GA 3' R 5' AGG ACG GTG CGG TGA GAG TG 3'

FV, factor V; *FII*, prothrombin; *PLA1/A2*, platelet glycoprotein IIb/IIIa; *MTHFR*, methylene tetrahydrofolate reductase

and 67bp, whereas in wild type of *FV*, three fragments were produced (163, 67 and 37 bp). A 345bp fragment of prothrombin gene was amplified and digested with *HindIII* restriction enzyme for detection of *FII* 20210G/A polymorphism. There is no cleavage site in wild type whereas mutated allele produces two fragments of 322 and 23bp. A 264 bp fragment was amplified and digested to two fragments (222 and 42 bp) by *MspI* restriction enzyme in *PLA1* polymorphism, whereas digestion of *GpIIIa* 1565T/C resulted in three fragments (173, 49, and 42bp). For identification of *MTHFR* 677C/T polymorphism a 198 bp fragment was amplified and digested by *HinfI* restriction enzyme in to two fragments of 175 and 23bp.

Statistical analysis: Statistical analysis was performed using SPSS (SPSS Inc., Chicago, USA). Descriptive analysis was used to describe patients' characteristics. Odds ratio (OR) was used to describe the strength of association between genetic polymorphisms and PE or DVT. Fisher's exact test was used to make comparisons among the groups.

Results

A total of 95 patients (41 males and 54 females) with VTE entered in to the study. There was neither significant difference in the age among PE, DVT/PE, and DVT patients (53.78±18.69, 49.01±21.20, and 48.03±18.30 yr, respectively) nor between males and females. (35.1% of PE, 52.2% of DVT/PE, and 45.7% of DVT patients were male). Distal DVT was

diagnosed in 31 patients and proximal DVT in 27. The most frequent transient risk factor prior to thrombosis in DVT was immobilization. In female patients with DVT, using contraceptive was the first acquired risk factor followed by immobilization. Surgery was the most frequent transient risk factor (47.4%) before PE episodes. No significant difference was seen in the family history among the three groups. Recurrent events were diagnosed in two (5.2%) of PE patients, six (26.1%) of PE/DVT, and 13 (37.1%) of DVT.

Investigated thrombophilia abnormalities: The presence of inherited thrombophilia abnormalities are shown in Table II. Overall, 22 polymorphisms were found in 37 PE patients and 15 polymorphisms in 23 DVT/PE patients compared to 35 polymorphisms in 35 DVT patients (1 per patient). The mean was 0.49 in the control group ($P<0.05$). Patients with DVT had more than four times of investigated genetic polymorphisms than control participants (OR: 4.52, 95% CI: 1.99-10.2). Such difference was not seen between the other two groups of patients and control group. There were more homozygous polymorphisms in DVT patients compared to PE and DVT/PE and the control group. Thirteen patients had coexistence of two polymorphisms.

Table III compares the presence of each polymorphism among the four groups. No significant difference was found in the occurrence of *FV*1691GA, *FII* 20210G/A, and *MTHFR* 677C/T among the four

Table II. Comparison of the total number of polymorphisms between patients and control participants

		Number of polymorphisms (%)					
		0	1	2	3	Mean polymorphism/person	
Groups (number of cases)	PE (37)	18 (48.6)	16 (43.2)	3 (8.2)	0	0.59	
	DVT/PE (23)	10 (43.5)	11 (47.8)	2 (8.7)	0	0.61	
	DVT (35)	8 (22.9)	19 (54.2)	8 (22.9)	0	1.0	
	Control (306)	174 (57.0)	114 (37.4)	17 (5.6)	1 (0.3)	0.49	
		Number of homozygous polymorphisms (%)					
		0	1	2	3	Mean polymorphism/person	
Groups (number of cases)	PE (37)	35 (94.6)	2 (5.4)	0	0	0.11	
	DVT/PE (23)	20 (87.0)	3 (13.0)	0	0	0.17	
	DVT (35)	24 (68.6)	10 (28.6)	1 (2.8)	0	0.34	
	Control (306)	290 (95.1)	15 (4.9)	0	0	0.05	

PE, pulmonary embolism; DVT, deep vein thrombosis
 $P<0.05$ (multiple comparisons among the groups)

Table III. Presence of investigated thrombophilia polymorphisms in the study groups

Groups (number of cases)	Polymorphisms			
	Number of affected cases (%)			
	<i>FV</i> 1691G/A	<i>FII</i> 20210G/A	<i>MTHFR</i> 677C/T	<i>GpIIIa</i> 1565T/C
PE (37)	2 (5.4)	1 (2.7)	14 (37.8)	5 (13.5)
DVT/PE (23)	1 (4.3)	0	11 (47.8)	3 (13.0)
DVT (35)	2 (5.7)	0	18 (51.4)	15 (42.9)
Control (306)	7 (2.3)	3 (1.0)	110 (35.8)	31 (10.2)
<i>P</i> value	0.21	0.66	0.24	<0.001

FV 1691G/A, factor V Leiden; *FII*, prothrombin; *MTHFR*, methylene tetrahydrofolate reductase; *GpIIIa*, platelet glycoprotein GPIIIa; PE, pulmonary embolism; DVT, deep vein thrombosis; Fisher exact test (4×2 table for each polymorphism)

groups, but a significant difference was seen in *GpIIIa* 1565T/C ($P<0.001$). In comparison between patients, *GpIIIa* 1565T/C polymorphism was found to be more than four times higher in DVT patients compared to PE and DVT/PE (OR: 4.80, 95% CI: 1.51-15.25 and OR: 5.00, 95% CI: 1.250-19.99, respectively). Such difference was not seen in other polymorphisms between PE and DVT/PE patients, PE and DVT, and DVT/PE and DVT (data not shown in Table).

Table IV shows comparison of different polymorphisms individually between the three groups of patients and the control group. No significant difference was observed between any group of patients and the control group in the frequency of *FV* 1691G/A. Only one case of *FII* 20210G/A mutation was seen in PE patients, but not in DVT and DVT/PE, and three in the control group, with no significant difference. The frequency of *MTHFR* 677C/T polymorphism in patients subgroups was not significantly different from the control group. The prevalence of homozygosity of the polymorphism was significantly higher in DVT patients but not PE and DVT/PE, compared with the control group ($P=0.02$, 0.22, and 0.25, respectively). Of the 35 DVT patients, 15 carried *GpIIIa* 1565T/C polymorphism, significantly higher compared with the control group ($P<0.001$, OR: 6.65 and 95% CI; 3.09-14.30). No difference was seen in the frequency of this polymorphism between the other two groups and the control group.

Discussion

DVT and PE are usually considered to be similar in the context of thrombophilia. However, data on some of these polymorphisms are not consistent. In this study, we compared these two clinical manifestations

of VTE and demonstrated some differences. Some of the investigated polymorphisms were associated with DVT but not with DVT/PE or PE alone. Our data demonstrated that the majority of patients showed single or multiple thrombophilic defects. Patients with DVT had significantly higher number of investigated thrombophilic polymorphisms compared with the control and the other two groups. This finding may support the hypothesis that thrombophilia leads to more stable clots which are less likely to detach and to make embolism. Previous data on the risk of PE or DVT associated with the presence of *FV*1691A mutation are controversial. Rahimi *et al*¹³ have reported the association between *FV*1691G/A mutation and DVT in Kurdish population in western Iran. Such divergence may be related to different backgrounds of Kurdish patients and ours. Gohil *et al*¹⁴ reported a prevalence of 23.5 per cent in PE patients while Biswas *et al*¹⁵ from India reported 10.3 per cent prevalence in DVT patients, both significantly higher compared to the control group. The homozygous *FV* 1691G/A has been found with more severe thrombotic phenotype, but the heterozygous with lower thrombotic risk¹⁶. Grifoni *et al*¹⁷ found a higher prevalence in DVT compared to the control group, but not a significant difference between PE patients and the control group. The prevalence was significantly higher in DVT and PE patients in Turkey^{18,19}, but not in Chinese/Thai patients¹⁴. Although we found the frequency of the polymorphism two times higher in our DVT patients, it did not reach to a significant level, which could be related to the lower number of patients or different ethnic background. Our data did not support the hypothesis of *FV*1691G/A paradox²⁰ as we did not find any association between DVT and *FV* 1691G/A.

Table IV. Comparison of inherited thrombophilia polymorphisms between each group of patients and the control group

Inherited thrombophilia polymorphisms	PE patients (n=37)	DVT/PE patients (n=23)	DVT patients (n=35)	Control (n=306)
<i>FV</i> 1691G/A				
Number of cases (%)	2 (5.4)	1 (4.3)	2 (5.7)	7 (2.3)
OR (95% CI)	2.59 (0.52-12.98)	1.94 (0.23-16.50)	2.59 (0.52-12.98)	
<i>FII</i> 20210G/A				
Number of cases (%)	1 (2.7)	0	0	3 (1)
OR (95% CI)	2.8 (0.28-27.69)			
<i>MTHFR</i> 677C/T				
Number of cases (%)	14 (37.8)	11 (48.7)	18 (51.4)	110 (35.9)
OR (95% CI)	1.09 (0.54- 2.19)	1.63 (0.70-3.82)	1.89 (0.93-3.81)	
<i>MTHFR</i> 677C/T (homozygote)				
Number of cases (%)	3 (8.1)	2 (8.7)	5 (14.3)	12 (3.9)
OR (95% CI)	2.16 (0.58-8.04)	2.33 (0.49-11.2)	4.08 (1.35-12.38)	
<i>GpIIIa</i> 1565T/C				
Number of cases (%)	5 (13.5)	3 (13)	15 (42.9)	31 (10.1)
OR (95% CI)	1.37 (0.50-3.82)	1.33 (0.37-4.71)	6.65 (3.09-14.30)	

FV 1691G/A, factor V Leiden; *FII*, prothrombin; *MTHFR*, methylene tetrahydrofolate reductase; *GpIIIa*, platelet glycoprotein GPIIIa; PE, pulmonary embolism; DVT, deep vein thrombosis; OR, odds ratio; CI, confidence interval

Pertaining to *FII* 20210G/A and its incidence in patients with DVT or PE, the data are controversial and higher or similar prevalence has been reported compared to control group^{13,14,21,22}. The increased risk of PE has been found with *FII* 20210G/A mutation, but decreased risk with *FV* 1691G/A in Italy²⁰. No significant difference was seen in the mutations in our patients and control groups and the frequency was lower compared with that previously reported in Caucasians and some other ethnics^{5,10,14}. Our data were consistent with the studies which found no relationship between DVT or PE and this polymorphism^{13,22}. The thrombophilia risk factors which were significantly associated with DVT but not PE or DVT/PE in our study were *GpIIIa* 1565T/C polymorphism of platelet glycoprotein IIb/IIIa and homozygous *MTHFR* 677C/T. We have previously reported that patients with VTE have significantly higher prevalence of coinheritance of more than one polymorphism, which is reflected more clearly in *MTHFR* 677C/T/*GpIIIa* 1565T/C²³. Here we found the highest frequency of *MTHFR* 677C/T and *GpIIIa* 1565T/C in our DVT patients. *GpIIIa* 1565T/C polymorphism increases the affinity of IIb/IIIa platelet receptor to fibrinogen and makes more aggregation as a baseline for thrombosis⁸.

In this case the clots may be more stable and adherent to the vessel wall and are less likely to detach, resulting in PE. Our data were not in agreement with Ivanov *et al*²² who found *GpIIIa* 1565T/C polymorphism higher in PE patients than in control group. Such discrepancy may be related to the ethnicity as well as risk factors and environment which equally influence the carriers and non-carriers. The association of polymorphism with arterial thrombosis has been reported in some studies^{24,25}.

The important finding of the present study was the prevalence of homozygous *MTHFR* 677C/T in DVT patients. The polymorphism results in a modest increase in homocysteine in plasma which may have a pathogenic significance in thrombosis^{26,27}. Hyperhomocysteinemia may not be a direct cause of thrombosis but a marker of systemic or endothelial stress and platelet activation²⁸.

We only studied four thrombophilia polymorphisms which could be considered as a limitation of our study. The other thrombophilia polymorphisms may have a role in PE pathogenesis. Inherited additional unknown or known risk factors may contribute to thrombosis in PE patients. In addition, limited number of patients was another limitation of our study.

In conclusion, our data indicated that *FV* 1691G/A and *FII* 20210G/A polymorphisms were not associated with PE, DVT/PE, and DVT in Central Iran. The *GpIIIa* 1565T/C polymorphism of *GP IIb/IIIa* and homozygous *MTHFR* 677C/T were associated with increased risk of DVT, but not PE and DVT/PE. Other thrombophilia risk factors may have a role in PE pathogenesis and should be investigated in this population. Because of different prognostic risk factors in PE and DVT, the treatment approach could be different.

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