

Effect of Platelet Glycoprotein Iib/IIIa PLA2 Polymorphism on Severity of Pulmonary Thromboembolism

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Background: Pulmonary thromboembolism (PTE) is among the leading causes of death following surgery and/or hospital admission. Role of thrombophilic risk factors in the etiology of PTE is well known; But not much data is available on their role in severity of PTE. The aim of this study was to investigate the role of thrombotic risk factors especially PLA2 polymorphism of platelet glycoprotein Iib/IIIa in the severity of PTE.

Materials and Methods: Genotyping from Factor-V (FVL) and prothrombin 20210A (PT20210A) mutations were shown to be significant risk factors for PTE and recurrent PTE. The plasma concentrations of platelet glycoprotein Iib/IIIa PLA2 polymorphism, presence of FVL and PT20210A mutations were studied in 37 patients with PTE.

Results: Eleven of these patients had recurrent PTE. Lung perfusion scans were scored according to the percentage of vascular obstruction. Patients who had a pulmonary vascular obstruction (PVO) score >50% were compared to those with PVO score <50%. There was no significant difference between patients with PVO score >50% and those with PVO score <50% with regard to the presence of FVL and PT20210A mutations. However, patients with PVO score >50% had a significantly higher frequency of platelet glycoprotein Iib/IIIa PLA2 polymorphism than those with PVO score <50%.

Conclusion: Our data suggest that presence of PLA2 is associated with an increased risk of PTE in the Iranian population. The association between recurrent events and coinheritance of more than one thrombophilic genetic risk factor shows that such carriers are at a higher risk of PTE.

Key words: Pulmonary thromboembolism, PLA2 polymorphism, Glycoprotein Iib/IIIa, Pulmonary vascular obstruction

INTRODUCTION

Pulmonary thromboembolism is a major health problem worldwide. The annual incidence of PTE in the general population of the Western countries is estimated to be 0.1-0.05% (1, 2). There are many well-recognized secondary risk factors for PTE. Primary risk factors, also known as congenital thrombophilic risk factors, should be seriously evaluated in patients with a documented, unexplained thrombotic episode. The most commonly

reported congenital risk factors are the factor-V 1691G-A (FVL) mutation, prothrombin20210A (PT20210A) mutation, protein-C and -S deficiency and anti-thrombin deficiency (3,4). GP IIIa is a highly polymorphic protein and platelet antigen 1 (PIA1) and 2 (PIA2) are its most common allelic isoforms. In PIA2 allele, cytosine replaces thymidine in exon2, which is phenotypically translated in the substitution of proline for leucine at position 33 of the mature GPIIIa (5). A previous in vitro study demonstrated

that the PLA2 variant enhanced the binding of GPIIb/IIIa receptor to fibrinogen and consequently increased platelet aggregation induced by agonists (6). The clinical impact of PLA2 polymorphism has been investigated in several diseases, in which thrombus formation is a key pathogenetic factor. Recently, a high frequency of platelet glycoprotein IIb/IIIa PLA2 polymorphism was shown to be an independent and dose-dependant risk factor for PTE and recurrent PTE (2, 7, 8). In this study, the authors aimed to investigate the role of some of the congenital thrombophilic risk factors (FVL, PT20210A mutation and high platelet glycoprotein IIb/IIIa PLA2 polymorphism frequency) in severity of PTE.

MATERIALS AND METHODS

Patients and controls

Thirty-seven unrelated patients, 13 males and 24 females, with a mean age of 53.78 years, were recruited from local volunteers with documented PTE and in the first step of research were compared with the control group of 152 unrelated healthy donors (matched by age and sex) with no history of vascular disease were recruited from local volunteers (2). They were recruited from Hajar Hospital, Shahrekord, Iran. Ethics committee of Shahrekord University of Medical Sciences approved this study (2). Patients provided written informed consent forms. Patients were clinically diagnosed using the Well's criteria and then PTE was confirmed in all patients by high-probability ventilation/perfusion lung scanning performed in Shahrekord Omid Center. In some patients (nearly 60%) spiral CT of the lung and mediastinum with contrast and with PTE protocol was performed in Shahrekord Hajar Hospital to rule out other diseases (2). Echocardiography was performed for all patients. Eleven of them (34.4%) were identified as having documented recurrent PTE from the hospital records. The inclusion criteria were the incidence of one or more episodes of pulmonary embolism at the time of diagnosis (9). Twenty-

three of the patients presented with isolated PTE and nine of which had PTE associated with DVT. Of the 37 patients studied, nine had no specific risk factor for PTE. In the remaining 28 patients, most had more than one risk factor, 15 had immobility, nine had DVT, nine had obesity, one had estrogen compound usage, and two had multiple trauma. None had malignancies or familial history of PTE or DVT. After diagnosis of PTE, blood samples were collected for laboratory studies. Twenty-nine patients received anticoagulation therapy with standard heparin, followed by warfarin. The remaining eight patients had clinically massive PTE and received thrombolytic therapy with streptokinase, followed by the same anticoagulation therapy. Written informed consent was obtained from each patient.

Study design

Perfusion-ventilation lung scans were performed in Shahrekord Omid Center. The clinicians obtaining the scans were unaware of the clinical condition of the patient at the time of diagnosis. To assess pulmonary vascular obstruction and severity of PTE, pulmonary vascular obstruction score (PVOs) was calculated by using a technique described by Meyer et al. (10) and Oguzulgen et al (11). We used this formula from Each lobe was assigned a weight based on the regional distribution of pulmonary blood flow in the supine position: right lower lobe 25%, right middle lobe 12%, right upper lobe 18%, left lower lobe 20%, lingula 12%, and left upper lobe 13%. Perfusion within each lobe was estimated from the anterior, posterior and oblique views. For each lobe, a semi-quantitative perfusion score, from 0-1 (0, 0.25, 0.5, 0.75 and 1), was estimated from the film density compared to the photodensity of an apparently normal perfused area. Each lobar-perfusion score was then calculated by multiplying the weight by the perfusion score. The overall perfusion score was determined by summing the six separate lobar-perfusion scores. The percentage of vascular obstruction by

perfusion scanning (PVOs) was then calculated as: PVOs (%)=(1-overall perfusion score) X100 (10,11).Patients with PVOs >50% were compared to those with PVOs<50%, with respect to the presence of FVL, PT20210A mutations and PLA2 polymorphism.

Pulmonary thromboembolism was clinically diagnosed according to the Well's criteria and was then confirmed by perfusion scan. Spiral CT of the lung and mediastinum with contrast and with PTE protocol was performed in 60% of patients with doubtful diagnoses.

Sample Collection and DNA Extraction:

Blood samples were collected from all patients at the time of diagnosis. Before the onset of anticoagulation therapy, venous blood was collected in 5 ml Vacutainer tubes with EDTA. DNA was isolated by the phenol chloroform method and quantified using Unico 2100 spectrophotometer. PCR using genomic DNA followed by restriction fragment length polymorphism (RFLP) were done to identify each polymorphism (Thermocycler ASTEC, PC818, Japan). Primers and restriction enzymes were provided by TAG, Copenhagen, Denmark and Fermentas, Russia, and summarized in Table 1. The thermal cycling conditions are summarized in Table 2. DNA sequencing was performed on selected samples to confirm the polymorphisms. The FVL mutate detected by the amplification of a 267-bp fragment using the specific primers. This fragment is digested with 1.5 units of MnII restriction enzyme to three fragments in wild type (163, 67 and 37 bp). The FVL mutation eliminates a restriction site and produces two fragments of 200 and 67 bp. For identification of FII G20210A polymorphism, a 345-bp fragment is amplified and digested with HindIII restriction Enzyme . There is no cleavage site in wild type, whereas the mutated allele produces two fragments of 322 and 23 bp. A 264-bp fragment is amplified and digested to two fragments (222 and 42 bp) by MspI restriction enzyme in PLA1 polymorphism. Introducing a new cleavage site in

PLA2 results in three fragments (173, 49 and 42 bp).To detect MTHFR C677T polymorphism, a 198-bpfragment is digested by Hinf I restriction Enzyme two fragments of 175 and 23 bp in the presence of MTHFR C677T polymorphism (12). However, there is no cleavage site in the un mutated allele. for diagnosis of PTE lung perfusion scan performed and then with formula of PVOs the percentage of vascular obstruction by perfusion scanning was calculated as:

PVOs (%)=(1-overall perfusion score)X100 (10,11).

Statistical analysis: Statistical analysis was performed using SPSS version 18. Descriptive statistics were employed for patients' clinical characteristics. The odds ratio (OR) was used to assess the strength of association between genetic polymorphisms and PTE. Statistical significance of genetic distribution as well as patients' age and sex was calculated using independent sample t-test (2).

Table 1. Restriction enzymes and primer sequences

Genetic polymorphism	Restriction enzyme	Primer sequences
FVL	MnII	F 5 TGC CCA GTG CTT AAC AAG ACC A 3 R 5 TGT TAT CAC ACT GGT GCT AA 3
FIIaG20210A	HindIII	F 5 TCT AGA AAC AGT TGC CTG GC 3 R 5 ATA GCA CTG GGA GCA TTG AAG C 3
PLA1/PLA2	MspI	F 5 TTC TGA TTG CTG GAC TTC TCT T 3 R 5 TCT CTC CCC ATG GCA AAG AGT 3
MTHFR C677T	HinfI	F 5 TGA AGG AGA AGG TGT CTG CGG GA 3 R 5 AGG ACG GTG CGG TGA GAG TG 3

FVL , Factor V Leiden; MethyleneTetrahydrofolatereductase (MTHFR), prothrombin

Regression was used to analyze the relationship between multiple independent polymorphisms and PTE. Mann-Whitney U test was performed for comparison of the number of mutations in patients with and without recurrent events and family history. All data of genetic factors were calculated in comparison to the control group.

Table 2. Thermal cycling conditions

Genetic polymorphism	Thermal cycling conditions
	96 °C for 5min
FVL	6 cycles of 96 °C for 1 min , 58°C for 1 min, 72 °C for 1 min 28 cycles of 96 °C for 30 s, 56° C for 30 s, 72 °C for 30s A final extension period of 5 min at 72 °C
FIIG20210A	94°C for 5min 33 cycles of 94°C for 30 s, 60°C for 45 s, 72°C for 60s A final extension period of 5 min at 72°C
PLA1/PLA2	96°C for 3min 5 cycles of 95°C for 3 min, 56°C for 1 min 31 cycles of 94°C for 40 s, 55°C for 40 s, 72°C for 40s A final extension period of 5 min at 72°C
MTHFR C677T	94°C for 5 min 5 cycles of 94°C for 60 s, 72°C for 1 min 25 cycles of 94°C for 30 s, 59°C for 40 s, 72°C for 30s A final extension period of 10 min at 72°C

FVL, Factor V Leiden; MethyleneTetrahydrofolatereductase (MTHFR)

RESULTS

Clinical characteristics of patients

Patients were recruited for this study from October 2009 to December 2010. Table 3 shows patients' characteristics (2).

Twenty-seven (37.5%) patients were under 45 years of age. Thirteen (35.1%) patients of the study population were men with a mean age of 48.38 years, whereas the mean age of 24 women (64.9%) was 53 years ($P=NS$). Patients with a previous history of PTE had a mean age of 47.6 ± 20.3 years compared with 52.1 ± 18.2 years in those without such history; no significant difference in this regard was observed ($P=0.42$); in addition, there was no significant difference between patients with family history of PTE and patients with no family history (mean age of 42.6 versus 52.3 years).

Investigated genetic factors

Twenty-four out of 37 (64.9%) patients compared with 132 out of 306 (43.2%) controls were carrier of at least one thrombophilia polymorphism. A total number of 57 mutations with a mean of 0.79 mutation were observed in

patients in contrast to 151 with a mean of 0.49 in controls ($P<0.001$). Table 4 summarizes data of investigated genetic factors. The prevalence of PLA2 was 27.8% in PTE patients versus 10.1% in controls (OR, 3.4; 95% CI, 1.08–6.44). In multivariate analysis with logistic regression, the OR for PLA2 was 3.43 ($P<0.001$).

Polymorphisms in patients with previous or family history of venous thromboembolism:

Seven (19.4%) patients had a previous history of PTE with 18 investigated polymorphisms compared with 37 polymorphisms in 57 patients without such history (2).

A total of 20 patients had PVO score $<50\%$ (54%) and 17 had PVO score $>50\%$ (46%). FVL and FIIG20210A mutations and MTHFR C677T and PLA2 polymorphism were present in two (5.4%), one (2.7%), 16 (43.2%) and 10 patients (27.02%), respectively. There was no significant difference between patients with PVO score $>50\%$ and those with PVO score $<50\%$ with respect to the presence of FVL [20% ($n=3$) and 17.6% ($n=3$) in patients with PVO score $>50\%$ and $<50\%$, respectively] and the presence of PT20210A mutation [6.7% ($n=1$) and 5.9% ($n=1$) in patients with PVO score $>50\%$ and $<50\%$, respectively].

Table 3. Patient characteristics

Characteristics of PE patients	
No. of patients	37
Mean age (years)	53.78
Sex	
Male N(%)	13(35.1%)
Female N(%)	24 (64.9%)
Positive family history N(%)	6 (16.2%)
Positive previous history N(%)	2 (5.4%)

PE: pulmonary embolism.

Patients with PLA2 polymorphism had more recurrent events than other patients (40 compared with 13.5%, $P=0.02$). One patient with a previous PTE history was a carrier of FVL and MTHFR C677T as homozygous.

Table 4. Inherited thrombophilia polymorphisms in patients with PTE.

Inherited risk factors	All patients with PTE (n=37)	Controls (n=306)
FVL		
Individuals (prevalence%)	2(5.4)	7(2.3)
OR (95% CI)	2.51(0.72-8.83)	
P-value	NS*	
FII G20210A		
Individuals (prevalence%)	1(2.7)	3(1)
OR (95% CI)	1.42 (0.146-13.88)	
P-value	NS*	
PLA2		
Individuals (prevalence%)	10 (27.02)	31 (10.1)
OR (95% CI)	3.4 (1.8-6.44)	
P-value	<0.001	
MTHFR C677T		
Individuals (prevalence %)	16 (43.2)	110 (35.9)
OR (95% CI)	1.43 (0.85-2.40)	
P-value	NS*	

CI, confidence interval; FVL, factor V Leiden; MTHFR:methylenetetrahydrofolatereductase; PE, pulmonary embolism; PTE, venous thromboembolism. * Not significant.

DISCUSSION

This study demonstrates that the prevalence of common inherited thrombophilic polymorphisms in our patients was neither similar to that in whites nor quite consistent with Asians. The findings of the present study show that some of the genetic thrombophilic polymorphisms enhanced the risk of PTE in our patients; however, the other investigated factors did not increase the risk. Among the patients with inherited thrombophilia, the highest prevalence was associated with having MTHFR C677T polymorphism (44.4%). The homozygous state of the polymorphism was significantly higher in patients than in controls (OR, 3.0;95% CI, 1.2-7.8), but no significant difference was seen in heterozygote carriers. The polymorphism is widespread among different populations, less frequent in some ethnic groups and more in others. It has been found in less than 2% of African-Americans and 11.2% of Australians, which is comparable with the rate obtained for Europeans (13-17). Our control group shows the homozygosity frequency of 3.9%, which is less than that of whites. Although the prevalence is slightly higher

in patients, we found no association between the polymorphism and PTE. This finding is different from the result of a study by Ivanov et al. (18) in which a lower prevalence in patients with pulmonary embolism was obtained. Polymorphism has been found to be equally distributed among thrombotic patients and controls in Turkey (12); however, it was identified, in agreement with our data, as a risk factor for VTE in Chinese/Thai populations (19). This polymorphism can increase the plasma level of Homocysteine and hyperhomocysteinemia may have a pathogenic significance in venous and arterial thrombosis (16, 20, 21). It has been recently suggested that hyperhomocysteinemia may not be a direct cause of thrombosis, but a marker of systemic or endothelial oxidant stress and platelet activation (22). Our study also demonstrated that the prevalence of FVL was higher in PTE patients than in controls (5.6 versus 2.3%); however, this difference did not reach a significant level. Many studies have indicated the role of FVL mutation in the cause of PTE. A prevalence of 23.5% has been found in patients with pulmonary embolism in Bulgaria (19), significantly higher than controls (23). Coppola et al. (24) and Biswas et al (25) found an association of homozygous FVL and some of the other rare abnormalities with more severe thrombotic phenotypes, but the common polymorphisms, such as heterozygous FVL, were associated with a lower thrombotic risk. Although we found the homozygous to be more prevalent in our patients in contrast to controls (2.8 versus 0.3%), the difference was not significant. The prevalence has been suggested to be significantly higher in thrombotic patients but not in young patients with coronary artery disease in Turkey (16, 26, 27). The mutation was not found to be in association with PTE in a Chinese/Thai population (19). Our data seem to be in agreement with their population rather than whites. This disparity between different cohorts could be related to not only the number of patients but also national risk factors such as higher BMI or smoking that influences the non carriers.

The prevalence of FII G20210A mutation was lower in our patients than that previously reported in whites and some other populations (5, 7, 26, 28, 29), as it was lower in non thrombotic controls as well (30, 31). Many studies have demonstrated the role of this polymorphism in the pathogenesis of PTE (5, 19); however, others found no relationship (19, 32). Our data are consistent with the latter, as we did not find a significant difference between patients and controls.

The most striking finding was that the PLA2 polymorphism was strongly associated with PTE ($P < 0.001$). The frequency was about three times higher in patients with PTE than in controls (27.8 versus 10.1%), and this polymorphism was the only independent one associated with PTE in a multivariate analysis ($P < 0.001$). Apparently, this polymorphism increases the affinity of glycoprotein IIb/IIIa receptor to fibrinogen and makes the platelets more susceptible to aggregation (2, 33), which could result in thrombosis. This finding is consistent with that of a study by Ivanov et al. (18) on patients with pulmonary embolism and indicated the contribution of PLA2 polymorphism to PTE pathogenesis. Although not many studies are available concerning the role of PLA2 allele in PTE (29), its role in arterial events has been shown in different studies (2, 34, 35).

As demonstrated in our study, the coinheritance of more than one investigated genetic polymorphism increased the risk of PTE. Patients with more than one inherited risk factors had also more recurrent events.

Although we did not find a significant difference between the age of patients and controls, the coinheritance of more than one inherited risk factor in patients with recurrent events influenced the age of patients (32 compared with 51.1 years in all patients). This finding is consistent with that of Ivanov et al. (18) who found the first event in patients with pulmonary embolism with coinheritance of more than one risk factor before 45 years of age. However, we did not find the FVL/PLA2 coinheritance to be more prevalent compared with their findings (18), which could be related to the lower

prevalence of the FVL mutation in our patients. Risk of recurrent PTE events has been found to be more prevalent in patients with FVL or FII G20210A mutation (35). Our data indicate that patients with a previous history of VTE have more thrombophilia mutation than those without such history.

This is in agreement with those finding a higher risk of recurrence in thrombophilia carriers (18, 25). Previous studies have evaluated the risk of PTE in family members of patients. Couturaud et al. (36) and Pourghysari et al. (2) found the risk of thrombosis to be higher in the relatives of unprovoked PTE patients with FVL or FII G20210A mutation than in those without such mutations. We did not find a significant difference in the number of mutations in patients with and without the first-degree family history (data not shown). This finding can be related again to the lower prevalence of FVL and FII G20210A polymorphisms in our population.

In this study, PVO score was used to assess the severity of PTE, which was found to be a reliable method of assessing the percentage of PVO (5). It was found that patients with PVO score $> 50\%$ had significantly higher concentrations of factor VIIIc. It was also shown that patients with high factor-VIIIc concentrations (> 150 IU) had an increased risk of experiencing severe PTE (PVOs $> 50\%$) (OR: 9.6; 95% CI: 1.8–49.4). In the present study, the prevalence of FVL and FII G20210A mutations was 14.9% but the prevalence of MTHFR C877T and PLA2 polymorphism was 26%, which are higher than the reported frequencies for healthy Turkish adults (10% and 2.6%, respectively) (1). The data on PTE patients were higher than previous reports (3, 4), but were not associated with the severity of PTE.

To date, there is no consensus about the duration of oral anticoagulant therapy in patients with high factor-VIIIc concentrations. The authors believe that because a high factor-VIIIc concentration plays a distinct role in the recurrence of VTE and the severity of PTE, prolonged anticoagulation must be taken into consideration. In patients with PLA2 polymorphism, we recommend anti

platelet aggregation therapy after at least six months of anti coagulation therapy (2).

CONCLUSION

Our data suggest that presence of PLA2 is associated with an increased risk of PTE in the Iranian population. The association between recurrent events and coinheritance of more than one thrombophilic genetic risk factor shows that such carriers are at a higher risk of PTE. The events can occur in patients at younger ages. The clinical implication of this study concerns the carriers of PLA2, perhaps requiring a management different from that of other patients. Awareness of existing genetic risks could provide a prophylactic advantage regarding the control of acquired risk factors. Furthermore, testing for FVL or FII G20210A did not have the ability to predict the risk of thrombosis in this population. Lung perfusion scans were scored according to the percentage of PVO (32) (patients who had a PVO score > 50% were compared to those with (PVO score <50%). There was no significant difference between the patients with PVO score >50% and those with PVO score <50%, with regard to the presence of FVL and PT20210A mutation. However, patients with PVO score >50% had significantly higher platelet glycoprotein IIb/IIIa PLA2 polymorphism than those with PVO score <50%.

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Conflicts of interest

The authors of this manuscript declare no competing financial interests.

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