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Original paper

IMPACT OF ACQUIRED AND GENETIC FACTORS ON THROMBOPHILIC PHENOTYPE IN FV LEIDEN MUTATION CARRIERS

Valentina Đorđević[1], Ljiljana Rakićević[1], Predrag Miljić[2], Danijela Miković[3], Mirjana Kovač[3], Dragica Radojković[1], Ana Savić[1]

[1]Institute of Molecular Genetics and Genetic Engineering, Belgrade, Serbia and Montenegro
[2]Institute of Hematology, University Clinical Centre of Serbia, Belgrade, Serbia and Montenegro
[3]Hemostasis Department and Hemophilia Centre, National Blood Transfusion Institute, Belgrade, Serbia and Montenegro

Summary: FV Leiden mutation is an important genetic risk factor for venous thromboembolsm (VTE). In this study we have analyzed clinical manifestation and the impact of other genetic and acquired risk factors in 100 patients (95 heterozygous and 5 homozygous) carriers of FV Leiden mutation. Among these patients, 91 experienced VTE, with down limb deep vein thrombosis as the most frequent manifestation. An acquired risk factor was present in 68.6% of women, whereas this was the case in 28.6% of men. FIIG20210A was present in 9.5%, MTHFR 677TT in 8.4% and both mutations in 2.1% of the heterozygous FV Leiden carriers. Our results suggest that knowledge of coexisting factors predisposing to VTE is very important for FV Leiden mutation carriers and may contribute to the prevention of VTE episodes.

Key words: FV Leiden, venous thromboembolism, FII G20210A, MTHFR C677T

Introduction

Thrombosis is multicausal disease in which both acquired and genetic influences may play important roles (1). The most important acquired risk factors are: age, trauma, surgery, immobilization, pregnancy, oral contraceptives and malignancy (1, 2).

The most frequent genetic risk factor known to date for venous tromboembolism is the Factor V G1691A mutation. Other frequent genetic risk factors include: prothrombin G20210A (FII G20210A) and methylenetetrahydrofolate reductase C677T (MTHFR C677T) mutations (2). Human coagulation factor V (FV) is a 300 kDa multidomain glycoprotein. In plasma FV is converted first to its active form (FVa), which has a very important role in haemostasis. FVa acts as a cofactor in the conversion of prothrombin to thrombin in the coagulation process (3). Its activity is down-regulated by activated protein C (APC), which inactivates FVa by proteolysis at Arg^{306}, Arg^{506} and Arg^{679}. FVa is first cleaved at Arg^{506}, and this peptide bond cleavage is essential for the optimal exposure, of cleavage sites of Arg^{306} and Arg^{679} (4). Reduced sensitivity of the anticoagulant effects of APC (APC resistance) increases thrombin generation and enhances blood coagulation. APC resistance is the most frequent genetic risk factor for thrombosis known to date (5). One possible mechanism for APC resistance is an impaired ability of APC to cleave FVa because of amino-acid substitutions within FV at the cleavage sites. The most prevalent substitution is at Arg^{506} caused by point mutation in FV gene G1691A, denoted as FV Leiden. This defect is associated with APC resistance in 95% of cases, and the rate at which mutant FV is inactivated by APC is 10- to 20-fold lower then normal (6). The FV Leiden mutation is present in approximately 3–7% of the Caucasian population, but is very rare in non-Caucasians (7, 8). This frequency is increased in patients with venous thromboembolism (VTE) to 15–50% (9, 10). The heterozygous state of FV Leiden mutation is associated with fivefold to 10-fold increase

Address for correspondence:

Valentina Đorđević
Institute of Molecular Genetics and Genetic Engineering
Vojvode Stepe 444A, P.O.Box 23
11010 Belgrade, Serbia and Montenegro
Tel: +381 11 3976658
Fax: +381 11 3975808
e-mail: qwert@eunet.yu

in risk of VTE and with 50- to 100-fold increase for the homozygous state (6, 10). The most common clinical manifestation of FV Leiden mutation is deep vein thrombosis (DVT) with or without pulmonary embolism (PE) (10, 11). Data to support its role in arterial thrombosis and recurrent miscarriages are less conclusive and future investigation are required (1, 12).

The G to A substitution at position 20210 of the 3'-untranslated region of the prothrombin gene is associated with elevated plasma prothrombin level and has been found it in approximately 6% of Caucasians with venous thrombosis (13).

The MTHFR C677T mutation is associated with hyperhomocysteinemia, which is recognized as a risk factor, but the relation with thrombosis remains unclear (14, 15).

So far, FV Leiden mutation has been investigated in many studies, but there is limited data for Serbian population (16, 17). Aim of this study was to analyze the clinical feature in a cohort of FV Leiden mutation carriers, and to assess how the co-inherence of the FV Leiden mutation with other genetic and/or acquired risk factors contributes to the thrombophilic phenotype observed in these patients.

Subjects and Methods

Subjects

A study was carried out in a group of 100 patients, FV Leiden mutation carriers. The patients were admitted to the Institute of Molecular Genetics and Genetic Engineering between 1999. and 2003. They were referred for genetic testing after objectively diagnosed venous or arterial thrombosis or after two or more spontaneous abortions. Information on the thromboembolic disease and risk factors was obtained from patient document files and personal interviews.

Methods

Sodium-citrate anticoagulant blood (5 mL sample) was obtained from each patient by peripheral venepuncture.

The FV Leiden and FII G20210A mutations were detected by multiplex polymerase chain reaction (PCR) on whole blood, followed by simultaneous digestion with specific restriction enzymes *MnlI* and *HindIII* (New England BioLabs) (18). The fragment of the MTHFR gene that included nucleotide 677 was amplified by PCR on whole blood, followed by digestion with *HinfI* (New England BioLabs) endonuclease. We used previously described primers (15) with the following modification of the PCR: PCR reactions were performed in a 25 μL final volume containing 50 mmol/L KCl, 100 mmol/L Tris-HCl, pH 9.0, 0.1% Triton X-100, 200 mmol/L dNTPs, 5 mmol/L $MgCl_2$, 10 pmol of forward and reverse primers and 1 L of whole blood. The thermal cycle profile was: 5 cycles consisting of 98 °C for 3 minutes and 55 °C for 3 minutes, to assure complete denaturation of DNA. One unit Taq polymerase (AppliedBiosystems) was added at 50 °C and after 5 minutes, 35 cycles consisting of denaturation at 94 °C for 30 seconds, annealing at 61 °C for 30 seconds and polymerization at 72 °C for 30 seconds were applied. Final extension of PCR products was at 72 °C for 10 minutes. The PCR reactions were performed in Mastercycler gradient apparatus (Eppendorf).

Normal and mutated alleles were distinguished by the size of the restriction fragments, using electrophoresis on 10% polyacrylamide gels and visualised by silver staining (19).

The statistical analysis was performed using Statistical Package for Social Science (SPSS).

Results

The main characteristics of the study group are shown in *Table I*. Of the 100 patients 95 (61.1% women and 38.9% men) were heterozygous and 5 (60% women and 40% men) were homozygous FV Leiden carriers. Among them, 78.9% heterozygous and all of homozygous FV Leiden carriers had suffered at least one VTE episode. Nine heterozygous carriers were without VTE (3 with arterial thrombosis and 6 women with miscarriages). Mean age of the first VTE episode was similar in all groups of patients (*Table I*).

The characteristics of VTE are listed in *Table II*. In most of the patients the thrombotic event was down limb DVT, with or without PE. Unusual manifestations of VTE were documented in nine patients (four of them suffered of upper limb DVT). Five heterozygous carriers experienced down limb DVT and other manifestation of VTE, two patients have down limb DVT and spontaneous abortion, and one patient had down limb DVT, PE and myocardial infarction.

In 31.4% female heterozygous carriers of FV Leiden mutation VTE developed without any acquired risk factors, whereas this was the case in 71.4% of males. In homozygous carriers these numbers were 33.3% and 50.0%, respectively. Pregnancy and puerperium were the most frequent risk factors (in 26 heterozygous and 2 homozygous carriers). Surgery and trauma were risk factors in 17.6% of women and 22.8% of men heterozygous for FV Leiden mutation. Other acquired risk factors were documented in only five patients (*Table III*).

Data on the prevalence of the FII G20210A and MTHFR C677T mutations are shown in *Table IV*. Among 95 heterozygous carriers of FV Leiden mutation coexistence with heterozygosity for FII G20210A and/or homozygosity for MTHFR C677T mutations (MTHFR 677TT) was detected in 20%. Only one patient was homozygous for both FV Leiden and

Table I Characteristics of patients with FV Leiden mutation

	FV Leiden heterozygous			FV Leiden homozygous		
	Women	Men	Total	Women	Men	Total
n	58	37	95	3	2	5
Mean age (range)	36.8 (20–63)	34.4 (1–70)	35.9 (1–70)	35 (19–57)	34 (33–35)	34.6 (19–57)
Patients with VTE, n (%) Mean age of the first VTE episode (range)	51 (87.9) 33.1 (19–61)	35 (94.6) 31.5 (1–59)	86 (90.5) 32.4 (1–61)	3 (100) 34.3 (18–56)	2 (100) 28.5 (23–34)	5 (100) 32.0 (18–56)
Patients with recurrent VTE, n (%)	8 (15.7)	6 (17.1)	14 (16.2)	0	0	0
Patients without VTE, n (%) – arterial thrombosis – recurrent miscarriages – HELLP syndrome	7 (12.1) 1 5 1	2(5.4) 2	9 (9.5) 3 5	0	0	0

VTE-venous thromboembolism

Table II Characteristics of venous thromboembolism in patients with FV Leiden mutation

Site of VTE	FV Leiden heterozygous		
	Women n = 51	Men n = 35	Total n = 86
Down limb DVT, n (%)	34 (66.7)	16 (45.7)	50 (58.1)
Down limb DVT and PE, n (%)	7 (13.7)	9 (25.7)	16 (18.6)
Isolated PE, n (%)	2 (3.9)	1 (2.9)	3 (3.5)
Upper limb DVT, n (%)	2 (3.9)	2 (5.7)	4 (4.7)
Renal vein thrombosis, n (%)	0	1 (2.9)	1 (1.2)
Cerebral vein thrombois, n (%)	0	1 (2.9)	1 (1.2)
Caval vein thrombosis, n (%)	1 (2.0)	1 (2.9)	2 (2.3)
Mesenteric vein thrombosis, n (%)	1 (2.0)	0	1 (1.2)
Other, n (%)	4 (7.8)	3 (8.6)	7 (8.2)
– down limb DVT and SA – down limb DVT and cerebral vein thrombosis – down and upper limb DVT and PE – down limb DVT, PE and MI	2 1 1	 1 2	
	FV Leiden heterozygous		
	Women n = 3	Men n = 2	Total n = 5
Down limb DVT, n (%)	2 (66.7)	2 (100)	4 (80.0)
Down limb DVT and PE, n (%)	1 (33.3)	0	1 (20.0)

DVT-deep vein thrombosis; PE-pulmonary embolism; SA-spontaneous abortion; MI- myocardial infarction

Table III Acquired risk factors associated with VTE episodes in patients with FV Leiden mutation

Acquired risk factors	FV Leiden heterozygous			FV Leiden homozygous		
	Women n = 51	Men n = 35	Total n = 86	Women n = 3	Men n = 2	Total n = 5
None, n (%)	16 (31.4)*	25 (71.4)*	41(47.7)	1 (33.3)	1(50.0)	2 (40.0)
Surgery, n (%)	7 (13.7)	4 (11.4)	11 (12.8)	0	0	0
Trauma, n (%)	2 (3.9)	4 (11.4)	6 (7.1)	0	0	00
Immobilization, n (%)	0	1 (2.9)	1 (1.2)	0	1 (50.0)	1 (20.0)
Pregnancy, n (%)	14 (27.5)	–	14 (16.3)	2 (66.7)	–	2 (40.0)
Puerperium, n (%)	12 (23.5)	–	12 (14.0)	0	–	0
Malignancy, n (%)	0	1 (2.9)	1 (1.2)	0	0	0

* $p<0.0001$

Table IV Coinheritance of FII G20210A and MTHFR C677T mutations in FV Leiden mutation carriers

	FV Leiden heterozygous			FV Leiden homozygous		
	Women	Men	Total	Women	Men	Total
n	58	37	95	3	2	5
FII G20210A, n (%)	6 (10.3)	3 (8.1)	9 (9.5)	0	0	0
Mean age of the first VTE episode (range)	26.7 (23–31)	22.7 (18–29)	25.3 (18–31)	–	–	–
MTHFR 677TT, n (%)	4 (6.9)	4 (10.8)	8 (8.4)	0	1 (50.0)	1 (20.0)
Mean age of the first VTE episode (range)	24.0* (20–26)	39.2* (34–40)	31.6 (20–40)	–	34	34
FII G20210A and MTHFR 677TT, n (%)	1 (1.7)	1 (2.7)	2 (2.1)	0	0	0
Mean age of the first VTE episode (range)	38	29	33.5 (29–38)	–	–	–

*p=0.028

MTHFR C677T mutations. Mean age of the first VTE episode was lower in the patients with coinheritance of two mutations, except for the group of men heterozygous for FV Leiden and homozygous for MTHFR C677T mutations. The difference between mean age of the first VTE episode for women and men heterozygous for FV Leiden and homozygous for MTHFR C677T mutation (24 and 39.2 years, respectively, p=0.028), was observed.

Discussion

The results of this study are the first data on the clinical features of thrombophilia in patients with FV Leiden mutation in our population. No difference between mean ages of the first VTE episode, between women and men was observed. Surprisingly, there was no difference between heterozygous and homozygous carriers, too. It is important to note that only five homozygous FV Leiden carriers were observed in this study, including female patient who suffered from first VTE episode at the age of 56 years. If we do not take this patient into account, mean age of first VTE episode for homozygous carriers was 26 years (range 18–34), which is almost 6 years less than in heterozygous carriers.

Among our patients, five female heterozygous carriers had recurrent miscarriages without any other established risk factors. This data support possible role of FV Leiden mutation in pathology of pregnancy loss, which is in concordance with previously published data (1).

Down limb DVT with or without PE was the most prevalent thrombotic manifestation (in 78.8% patients), which is in concordance with previously reported data (9, 10).

We also evaluated frequency of the most important acquired factors for VTE known to date. In 71.4% of men none of known acquired risk factors were present and this was the case in 31.4% of women. This sex-related difference was statistically significant (p<0.0001). Therefore, it seems reasonable to suspect that there are still unknown triggering events for VTE development (infections or hormones balance, for example) in males. Large-scale epidemiological studies on this are required.

Prevalence of heterozygous carriers for FV Leiden, FII G20210A and homozygous carriers of MTHFR C677T mutation in healthy Yugoslav population was previously determined to be 5.4%, 4.6% and 11.5%, respectively (17). Based on this data, it can be speculated that 0.24% of healthy individuals are heterozygous for both FV Leiden and FII G20210A mutations, 0.61% are heterozygous for FV Leiden and homozygous for MTHFR C677T mutation and 0.03% are carriers of all three mutations. In this study observed frequencies was 40-fold, 14-fold and 70-fold higher, respectively. These findings confirm several previous reports, which in other populations described an enhanced risk of VTE in carriers of FV Leiden when associated with FII G20210A and MTHFR C677T mutations (20, 21). We found difference between mean age of the first VTE episode in women and in men heterozygous for FV Leiden and homozygous for MTHFR C677T mutation. (The first VTE occurred significantly earlier in women (mean age 24 years) than in men (mean age 39.2 years) p=0.028). Further studies on a larger cohort of patients are needed for conformation of our results.

In conclusion, our study supports the concept of thrombophilia as a multifactorial disease. FV Leiden mutation is important genetic risk factor for VTE. In majority of our patients, FV Leiden carriers, an additional acquired or genetic risk factor were present. The knowledge of coexisting factors predisposing to VTE is very important for FV Leiden mutation carriers and may contribute to the prevention of VTE episodes.

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UTICAJ STEČENIH I GENETIČKIH FAKTORA NA ISPOLJAVANJE TROMBOFILIJE KOD NOSILACA FV LEIDEN MUTACIJE

Valentina Đorđević[1], Ljiljana Rakićević[1], Predrag Miljić[2], Danijela Miković[3], Mirjana Kovač[3], Dragica Radojković[1], Ana Savić[1]

[1]Institut za molekularnu genetiku i genetičko inženjerstvo, Beograd, Srbija i Crna Gora
[2]Institut za hematologiju, Klinički centar Srbije, Beograd, Srbija i Crna Gora
[3]Institut za transfuziju krvi, Centar za ispitivanje poremećaja hemostaze, Beograd, Srbija i Crna Gora

Kratak sadržaj: FV Leiden mutacija je veoma značajan genetički faktor rizika za pojavu venskog tromboembolizma (VTE). U okviru ove studije analizirana je klinička slika i uticaj stečenih i genetičkih faktora rizika u grupi od 100 bolesnika, nosilaca FV Leiden mutacije (95 heterozigota i 5 homozigota). Devedeset jedan bolesnik je imao VTE, sa trombozom dubokih vena donjih ekstremiteta kao najčešćom manifestacijom. Kod 68,6% žena bio je prisutan neki od stečenih faktora rizika, dok je to bio slučaj kod 28,6% muškaraca. Mutacija FII G20210A je detektovana kod 9,5%, MTHFR 677TT kod 8,4%, a obe mutacije su bile prisutne u 2,1% heterozigotnih nosilaca FV Leiden mutacije. Rezultati ove studije ukazuju na značaj poznavanja udruženih faktora rizika kod pacijenata koji su nosioci FV Leiden mutacije.

Ključne reči: FV Leiden, venski tromboembolizam, FII G20210A, MTHFR C677T

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