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Research Article

Factor V Leiden, prothrombin G20210A and MTHFR C677T mutations in Romanian patients with deep venous thrombosis

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Abstract. Objective: to determine the frequency of factor V Leiden, prothrombin G20210A and MTHFR C677T polymorphisms in idiopathic deep venous thrombosis (DVT) and the associated risk. Materials and methods: an observational, case-control study was designed and 144 subjects were enrolled: 72 with confirmed idiopathic DVT of the lower limbs and 72 sex- and age- matched healthy controls. Results: the presence of at least one genetic mutation, found in 61.11% patients with DVT versus 44.44% controls was associated with almost double risk of DVT (OR=1.964, p= 0.045, 95% CI: 1.011-3.814). Only factor V Leiden, detected in 13.88% patients with DVT and 2.77% of controls, showed a significant association with DVT (p= 0.015, OR= 5.645, 95% CI: 1.190- 26.762). Prothrombin G20210A polymorphism was equally distributed in DVT and controls, 2.77%. MTHFR C677T polymorphism presented the highest prevalence, 58.97% in DVT and 44% in controls, but without significant difference (p= 0.498, OR= 1.257, 95% CI: 0.647-2.441). Among the mutant genotypes, only heterozygosity for factor V Leiden was associated with 8.875- fold increased risk of thrombosis (p= 0.015, 95% CI: 1.080-72.923). Conclusions: this study may be considered the first attempt offering some data regarding the frequency of genetic factors in Romanian patients with DVT and confirms the association of factor V Leiden with DVT in this population.

Key Words: deep venous thrombosis, factor V Leiden, protrombin G20210A, MTHFR C677T, thrombophilia

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Introduction

Deep venous thrombosis (DVT), with an incidence of about 1 case/year/1000 adults, is a multifactorial disease, result of the interaction between genetic and acquired risk factors. Although considered idiopathic in majority of the cases, an underlying cause could be detected in up to 80% of the patients with DVT, as shown in the literature (Whitlatch 2008). Genetic factors contribute also to the increased tendency towards coagulation- thrombophilia. The most common inherited thrombophilic factors in Caucasians are factor V Leiden (with a prevalence of 1-15% in healthy population, respective 15-65% in venous thromboembolism) and factor II (prothrombin) G20210A polymorphisms (found in 1-8% of healthy subjects, respective 3-17% in venous thromboembolism) according to Jadaon (2011a). Their frequencies vary according to geographical and ethnic factors.

Factor V Leiden is the result of the substitution of adenine to guanine at nucleotide position 1691 of exon 10 of FV gene, leading to procoagulation effects by the elimination of one of the cleavage sites for Activated Protein C (also called resistance to APC); most homozygotes for factor V Leiden experience at least one thrombotic episode in their life time, presenting 80-fold higher risk for DVT whereas heterozygotes, found in 15-20% of cases with venous thromboembolism, have 3- to 5-fold increased risk of thrombosis, according to Franchini (2012). Prothrombin G20210A polymorphism represents the substitution of adenine to guanine at the position 20210 in the regulatory 3'end of the prothrombin gene, leading to an increased efficiency of the 3′ end cleavage signal and a more effective poly(A) site, as shown in literature (Bosler et al 2006). The prevalence in Europe is of 1.7-3% amongst healthy individuals, with the highest in European Caucasians, and increases in DVT at 6.2%, respective 18% in the presence of a positive family history; the relative risk of DVT is 2-4- fold higher in carriers of this mutation and increases at 16- fold in patients presenting both factor V Leiden and factor II G20210A polymorphisms, as Jadaon has shown (2011b). Methylenetetrahydrofolate reductase (MTHFR) C677T polymorphism, the result of the alanine to valine substitution at codon 222, is associated with decreased activity of the enzyme 5,10 MTHFR and hyperhomocysteinemia, according to literature (Fay 2012). Although some studies showed the association of MTHFR C677T with DVT (Shmeleva et al 2003; Kupeli et al 2011), the current knowledge does not consider it as a significant risk factor, as it was shown by Spiroski et al (2008), Bezemer et al (2007), Naess et al (2009).

While numerous studies worldwide showed the prevalence and the thrombotic risk of these genetic factors, as far as we know, few Romanian data were previously reported, regarding only the pregnancy-associated thrombophilia (Trifa et al 2009; Popp et al 2012). We aimed to assess the frequency of factor V Leiden, prothrombin G20210A and MTHFR C677T polymorphisms in a group of Romanian patients with DVT and the associated risk.

Material and method

We designed a case-control, analytical, observational study, including 144 subjects admitted or visiting ambulatory an Internal Medicine Department of the University of Medicine and Pharmacy Cluj-Napoca, Romania. They were divided into 2 groups: the first group consisting of 72 patients with idiopathic DVT of the lower limbs and the second group, of 72 sex- and age- matched controls without thrombotic disease (according to Wells score and duplex ultrasound) selected from the subjects presenting for routine medical check-up. DVT was diagnosed based upon Wells score and duplex ultrasound; idiopathic thrombosis was defined by the DVT occurrence in the absence of a trigger risk factor (plaster cast immobilization and/ or fracture of a lower limb, surgery under general anesthesia, bed rest for more than three days, active cancer, pregnancy/post-partum, oral contraception/hormone replacement therapy in the last year, a journey lasting more than 6h) according to recent guidelines (Pernod et al 2009). All subjects presenting conditions predisposing to DVT (congestive heart failure, acute myocardial infarction, stroke, inflammatory bowel diseases, acute infection, obesity, varicose veins) were excluded from the study. The participants were enrolled after written informed consent and with the approval of the Ethic Committee of the University. For the ultrasound examination, a 3-5 MHz (for iliac veins) convex transducer and 5-10 MHz (from femoral level to distal) linear transducer (Aloka Premiere alpha10) were used.

Genetic polymorphisms were analyzed using PCR-RFLP (Polymerase Chain Reaction-Restriction Fragment Length Polymorphism) method. The commercial kit Wizard Genomic DNA Purification Kit (Promega®, USA) was used for the genomic DNA extraction, from 300 µL of peripheral blood. The PCR reactions were performed in a thermocycler, Mastercycler Personal (Eppendorf, Germany) in 25 µl reaction volume, containing 100 ng genomic DNA, 12.5 µl 2XPCR Master Mix (Fermentas®, Lithuania), 1 µl BSA solution 2 mg/ml (Bovine Serum Albumine, Fermentas®, Lithuania) and 6-10 pmoli of each primer (Eurogentec®, Belgium). The amplification products were digested at 37ºC, for 8-12 hours, with specific restriction enzymes: Mnl I for factor V Leiden, Hind III for factor II G20210A, respective Hinf I for MTHFR C677T (Fermentas®, Lithuania). The products of digestion were electrophoresed using 2% agarose gel (Agarose LE, Analytical Grade, Promega®, USA) and DNA fragments were coloured with ethidium bromide 10 mg/ml (Ethidium Bromide, Promega®, SUA) and then the gels were read with an UV transillumination system coupled with a photo camera (Vilber Lourmat Imaging System®, France) (Bertina et al 1994; Poort et al 1996; Zhou-Chun et al 2007). Factor V Leiden abolishes a restriction situs of the enzyme Mn1; thus, after the digestion of 267 pb (pair basis) amplicons, homozygotes for the normal allele present 3 fragments of 163, 67 and 37 pb; heterozygotes present 4 fragments of 200, 163, 67 and 37 pb, whereas homozygotes for mutant allele present 2 fragments of 200 and 67 pb, respectively. Mutation G20210A creates a restriction situs for the enzyme Hind III and after the digestion of 345 pb amplicons, the homozygotes for normal allele present the fragment of 345 pb, heterozygotes present 3 fragments of 345, 322 and 23 pb, whereas homozygotes for mutant allele present 2 fragments of 322 and 23 pb, respectively. MTHFR C677T mutation creates a restriction site for enzyme Hinf I. Thus, after the digestion of 265 pb amplicons with enzyme Hinf I, the normal genotype presents the full fragment of 265 pb; heterozygous genotype MTHFR C677T presents 3 fragments of 265, 171 and 94 pb and the homozygous mutant genotype MTHFR 677TT presents 2 fragments of 171 and 94 pb, respectively.

The descriptive statistics was used for the prevalence of genetic polymorphisms and bivariate inferential statistics was applied for the assessment of the association with DVT. The frequency of the genotypes in groups was compared using two-by-two contingency table and analyzed with chi-square χ^2 with Yates' correction test. Odds ratio (OR) with 95% confidence intervals (CI) showed the the strength of the association between genotype and thrombotic risk. The criterion for statistical significance was $P<0.05$. Statistical analysis was performed with the Statistical Package for the Social Sciences (SPSS) 16.0 for Windows.

Results

The mean age was 56.819 years old in DVT group (standard deviation 14.68), respective 56.305 years old in controls (standard deviation 10.26), without significant difference between groups (P=0.808); the male/female ratio in DVT and controls was 1.322, respective 1.482 (corresponding to 41 men and 31 women in DVT group, respective 43 men and 29 women in control group). The presence of at least one genetic mutation, found in 44 patients with DVT (61.11%) and 32 controls (44.44%) was associated with 1.964- fold increased risk of DVT (OR), p= 0.045, χ^2= 4.012, 95% CI: 1.011-3.814. The distribution of genetic factors did not differ significantly between sexes, being detected in 25 men and 19 women in DVT, respective in 18 men and 14 women in controls (P= 0.960, χ^2=0.002, OR= 1.023, 95% CI: 0.408-2.564).

Factor V Leiden was present in 10 patients with DVT (13.88%) and 2 controls (2.77%), showing a significant association with DVT: P= 0.015, χ^2= 5.818, OR= 5.645, 95% CI: 1.190- 26.762. Prothrombin G20210A polymorphism presented the lowest prevalence both in DVT and controls, 2.77%, and no significant association with DVT, P= 1, χ^2= 0.00, OR= 1, 95% CI: 0.137- 7.299. MTHFR C677T polymorphism was found in 32 patients (58.97%) and 28 controls (44%) without significant difference: P= 0.498, χ^2= 0.457, OR= 1.257, 95% CI: 0.647-2.441. The frequency and the association of all the mutant genotypes with DVT were analysed except for homozygous prothrombin G21210A polymorphism, since none of the patients and controls presented this genotype (table 1).

Among the mutant genotypes, only heterozygosity for factor V Leiden genotype was associated with 8.875- fold increased risk of thrombosis: p= 0.015, χ^2= 5.807, 95% CI: 1.080-72.923. A family history of DVT, found in 7 cases with at least one mutation and in 1 case without mutation, was associated with 6.797- fold increased risk of DVT, p= 0.042, χ^2= 4.097, 95% CI: 0.814-56.748.

A previous episode of venous thromboembolism in DVT patients with a genetic mutation (6 cases versus 1 case without mutation) was significantly associated with an increased risk of DVT, p= 0.050, χ^2= 3.753, OR= 6.454, 95% CI: 0.756-55.047. Patients carrying 2 genetic polymorphisms (found in 10 cases with DVT versus 3 controls) presented a 3.709- fold higher thrombotic risk, p= 0.041, χ^2= 4.143, 95% CI: 0.976- 14.097.

Table 1. The frequency of mutant genotypes and the association with DVT

Mutant genotype	(n=); (%) DVT (n=); (%) controls	χ^2	P	Odds Ratio	95%CI lower limit	95%CI upper limit
homozygous factor V Leiden	2; 2.78% 1; 1.39%	0.34	0.559	2.028	0.179	22.883
heterozygous factor V Leiden	8; 11.11% 1; 1.39%	5.807	0.015	8.875	1.08	72.923
heterozygous prothrombin G20210A	2; 2.77% 2; 2.77%	0	1	1	0.137	7.299
homozygous MTHFR 677TT	11; 15.28% 4; 5.56%	3.646	0.056	3.065	0.927	10.131
heterozygous MTHFR C677T	21; 29.17% 24; 33.33%	0.29	0.589	0.823	0.406	1.668

This corresponds to 46.59% increase risk comparatively to the presence of only one genetic factor. The compound genotypes in DVT were as follows: heterozygous MTHFR C677T/Leiden heterozygous, homozygous MTHFR 677TT/Leiden heterozygous (each with a frequency of 4.166%), heterozygous MTHFR C677T/Leiden homozygous (with a frequency of 2.777%), heterozygous MTHFR C677T/ heterozygous prothrombin G20210A and homozygous MTHFR 677TT/heterozygous prothrombin G20210A (each with a frequency of 1.388%). Due to the low frequency, the analyis of the associated risk of each compound genotype has not been performed.

Discussion

Factor V Leiden is a well-known risk factor for DVT and our study confirmed its role in Romanian patients. The frequency of 13.88% in DVT is close to those reported in Greece (16.8%) by Hatzaki et al (2003), Croatia (16%) by Alfirevic et al (2010), Italy (15.3%) by de Stefano (1999) and Turkey (15.2%) by Sahin et al (2012) and slightly lower than in other countries of Southeastern Europe (29.3% in Serbia, 25% in Bulgaria, 21.1% in Macedonia) as shown in literature (Djordjevic et al 2004; Boyanovsky et al 2001; Arsov et al 2006). A previous Romanian study regarding thrombophilia associated with pregnancy complications found a lower prevalence of factor V Leiden (8.3%), explained by the selection of the cases (Stamatian et al 2009). The prevalence of factor V Leiden in our control group (2.77%) is similar with the reported data in healthy Caucasians in Europe, 3-12% (Jadaon 2011a). Homozygous genotype was detected only in 2 patients with DVT and thus we found no significant association with thrombosis. The same low distribution was reported in our geographic area: 2.7% in Turkey, 2 cases amongst 190 patients in Macedonia, zero in Croatia (Sahin et al 2012; Arsov et al 2006; Alfirevic et al 2010). The risk of DVT in heterozygotes for factor V Leiden in our population was concordant to the literature, 8.8-fold versus 7-fold increased risk (Franchini 2012). Prothrombin G20210A mutation presented the lowest prevalence in our study, 2.77%, in both DVT and controls, similar with the reported data for Caucasians and from our geographic area, 3-24% in DVT, respective 1-12% in general population (Jadaon 2011; Bosler 2006; Alfirevic 2010). We found no homozygous genotype, as some reports from the region: Croatia, Turkey, Bulgaria (Alfirevic et al 2010; Sahin et al 2012; Boyanovsky et al 2001). In contradiction with the majority of the studies, we found no association of prothrombin G20210A mutation with DVT; however, a recent Croatian study (Alfirevic et al 2010) as well as an American study conducted on a large cohort showed similar findings, suggesting that sample size explains only partly these results (Ridker et al 1999). MTHFR C677T polymorphism presented the highest prevalence in DVT (58.97%) as reported in some of our neighboring countries: 54% in Croatia (Alfirevic et al 2010), 52.6% in Russia (Shmeleva et al 2003) and similar with a previous Romanian study regarding abortions associated with thrombophilia (59.5%). (Popp et al 2012). We found homozygous MTHFR 677TT genotype in 15.28% of DVT patients, concordant with Southeastern Europe data: 13% in Serbia, 16.4% in Macedonia, 17.8% in Greece (Djordjevic et al 2011; Spiroski et al 2008; Angelopoulou et al 2000). The association with DVT was very close to the statistical significance in our study. Although we found a high prevalence of heterozygous MTHFR C677T genotype (29.17% in DVT, 33.33% in controls), a slight lower than reported (34.5% in DVT, respective 34.3% in healthy people), there is no association with DVT, concordant with literature (Couturaud et al 2000).

We found the family history of DVT in patients carrying genetic mutation associated with 6.79 higher thrombotic risk, as shown by a population-based case-control study: the OR for DVT were 5.4 in case of a relative younger than 50 years and 17.8 when at least two relatives were affected (Bezemer et al 2009). Literature shows that family history, regardless of the other risk factors identified, may be more useful for the risk assessment than thrombophilia testing (Eikelboom et al 2011). Recent guidelines show that case finding of asymptomatic relatives with low risk thrombophilia, such as factor V Leiden or prothrombin mutation, is not indicated (recommendation grade 1B- a strong recommendation applying to most patients) (Baglin et al 2010). We found an increased risk for DVT recurrences in patients with genetic mutations, such is mentioned in the literature (Prandoni et al 2007; Kyrle et al 2010). However, there are still controversies, since some data failed to prove it

(Christiansen 2005; Baglin et al 2003). Up to 2012, no randomized controlled trials or controlled clinical trials comparing the rate of recurrent venous thromboembolism in DVT patients tested for thrombophilia versus those who were not tested had been found in literature (Cohn et al 2012).
The present study included a relative small sample of patients and future studies are required to confirm our findings. The assesment of deficiencies of protein C, S and antithrombin (rare, but strong genetic risk factors for DVT) has not been performed, representing another limitation of our study.

Conclusions

We confirmed the importance of factor V Leiden for DVT in our group of population; this study may represent the first attempt showing the frequency and the role of genetic factors in Romanian patients with DVT.

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