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Factor V Leiden, Prothrombin G20210A, and Protein C Mutation Frequency in Turkish Venous Thrombosis Patients

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Several inherited polymorphisms are associated with risk of venous thrombosis, including mutation at codon 506 of the factor V gene, mutation at position 20210 of the prothrombin gene, and mutations in the protein C gene. In this study, genotyping for factor V, prothrombin, and protein C mutations was performed in 50 patients and 25 control subjects by polymerase chain reaction–based analysis. The prevalence of factor V and prothrombin mutations was not significantly different from that in the general population. Nine of the patients had heterozygous protein C mutation. There was a high prevalence of the mutated protein C allele in the pulmonary emboli group (42.8%). Protein C mutation incidence was higher in the pulmonary emboli group than in the deep vein thrombosis (8.33%) and cerebral vein thrombosis (16.1%) groups. These results indicate that patients with protein C deficiency have a greater risk of thrombosis than patients with factor V or prothrombin G20210A mutation.

Keywords: factor V Leiden; protein C; prothrombin; venous thrombosis; coagulation factors

The obstruction of circulation by clots that are formed locally in veins or released from a thrombus elsewhere is called venous thrombosis.[1] Underlying causes that predispose individuals to thrombosis exert their effects by different mechanisms, some of which have a defined genetic basis. In the absence of genetic deficiencies, thrombosis occurs mainly in the older population or in the context of marked environmental influences such as surgery, trauma, immobilization, obesity, and pregnancy.[2] There is convincing evidence that hereditary abnormalities of the coagulation system are associated with an increased risk for both spontaneous and risk period–related venous thromboembolism.

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The main genetic factors that play a role in thrombosis are factor V, protein C, protein S, antithrombin, and fibrinogen.

Protein C is the key component of the natural anticoagulant pathway.[3] It is activated by thrombin bound to thrombomodulin on the surface of the endothelial cell. Protein C performs its anticoagulant function when it is converted to a serine protease by the thrombin-thrombomodulin complex or thrombin alone.[4,5] Genetic variations or mutations in the gene coding for protein C result in protein C deficiency. Patients homozygous for protein C deficiency or exhibiting compound heterozygosity for this anticoagulant factor have been found to be at increased risk for severe thrombotic disorders.[6-8]

Factor V circulates in the blood as an inactive cofactor. It can be activated by thrombin, resulting in the formation of a 2-chain molecule (Va) that serves as a cofactor of factor Xa during the conversion of prothrombin to thrombin. Factor Va is inactivated through selective proteolytic cleavages in its heavy chain at positions Arg 306, Arg 506, and Arg

679 by activated protein C. Recently, resistance to activated protein C has been described.[9] The biochemical and molecular basis for the resistance to activated protein C is explained by the inheritance of a mutation in the coagulation factor V gene (factor V Leiden [FVL]) in over 90% of patients. This mutation is caused by the substitution of a single amino acid, glutamine for arginine, at position 506.[10-13]

Prothrombin is the inactive precursor of thrombin, which is the end product of the coagulation cascade.[14] The prothrombotic mutation reported by Poort et al[15] is a guanine-to-adenine transition at position 20210 in the sequence of the 3′-untranslated region (UTR) of the gene encoding prothrombin. This mutation is found to be associated with increased prothrombin levels and a 3-fold risk of venous thrombosis.[15-17] No specific function has been defined for the 3′UTR.[15] However, there is accumulating evidence that novel mechanisms of gene regulation, including mutations in splice, donor, and promoter elements, may be important in regulating the gene expression.

The aim of this study was to investigate the role of FVL, prothrombin G20210A, and protein C mutations and to determine the frequency of coexistence of these mutations in venous thrombosis.

Materials and Methods

A total of 50 patients with venous thrombosis (28 men and 22 women; median age = 48.14 years; range = 19-78) and 25 control subjects (10 men and 15 women; median age = 32.4 years; range = 22-47) were studied. Inclusion criterion for the subjects was a documented history of thrombosis with 1 or more episodes. A total of 31 patients had cerebral vein thrombosis (CVT), 12 had deep vein thrombosis (DVT), and 7 had pulmonary embolism (PE).

Plasma Protein C Levels

The protein C antigen levels were measured by enzyme-linked immunosorbent assay using the Asserachrom Protein C Kit (Diagnostica Stago Co, Asnieres-Sur-Seine, France). Antigen levels were calculated relative to normal pooled plasma levels (100%). The normal range (mean ± 2 SD) was previously determined on normal subjects (0.70-1.4 for immunologic protein C).

Polymerase Chain Reaction and Single-Strand Conformational Polymorphism Analysis for Protein C

Genomic DNA was prepared from patients' whole blood as described previously. Exons and their flanking intron regions were amplified by polymerase chain reaction (PCR) except for exon 1. Exons 3-6 were amplified in 2 reactions. Exon 9 was amplified in 2 segments using 2 sets of primers. The sequences of the primers used in the study and PCR conditions are listed in Table 1. The final PCR reaction contained 0.5 to 1 μL of the template DNA, 200 μM deoxyribonucleotide triphosphate (dNTP), 10 pmol of each primer, and 0.5 U *Taq* polymerase in a total volume of 25 μL reaction buffer (50 mM KCl, 20 mM Tris-HCl, pH 8.3). The PCR products were diluted 3 to 5 times with 95% formamide in 10 mM NaOH, 0.05% bromophenol blue, and 0.05% xylene cyanol for single-strand conformational polymorphism (SSCP) analysis. The diluted samples (15 μL) were denatured at 95°C for 5 minutes, immediately chilled on ice, and loaded onto a 6% nondenaturing polyacrylamide gel containing 5% glycerol. Electrophoresis was performed at room temperature for 5 hours at 170 V. The bands were visualized by silver staining.

A fragment of the 3′UTR of the prothrombin gene was amplified by PCR. The PCR reaction mix contained 50 mM KCl; 10 mM Tris-HCl, pH 9.0; 1.5 mM $MgCl_2$; 200 μM of each dNTP; 150 ng DNA sample; 10 pmol of each primer (forward, 5′-ATTGATCAGTTTGGAGAGTAGGGG-3′; reverse, 5′-AATAGCACTGGGAGCATTGAAGCT-3′); and 2.5 U *Taq* polymerase. The reaction was begun by incubation at 93°C for 7 minutes followed by 35 cycles of denaturation at 94°C for 30 seconds, annealing at 60°C for 30 seconds, and extension at 72°C for 30 seconds, with a final extension at 72°C for 10 minutes.

The analysis of the FVL (1691G to A) allele was performed by amplification with the following oligonucleotide primers: forward, 5′-TCAGGCAGG AACAACACC-3′; reverse, 5′-GTTACTTCAAGGA-CAAAATACCTGTAAAGCT-3′. Polymerase chain reaction was performed in a 25-μL reaction mixture containing the same components as the prothrombin reaction except for $MgCl_2$ (0.5 mM). Amplification was performed for 35 cycles, with denaturation at 94°C for 1 minute, annealing at 58°C for 1 minute,

Table 1. The Sequences of Primers and Polymerase Chain Reaction (PCR) Conditions

Exon		Primers	$MgCl_2$ Concentration (mM)	Annealing, Heat (°C)/ Time (min)	Extension, Heat (°C)/ Time (min)	PCR Product (bp)
2		C1: 5′-CTAGGAATTCGCTCAGAAGTCCTCCTCAGA-3′ C2: 5′-GTACAAGCTTATGCCACCAGGGCCTTGGAG-3′	1.5	55/2	72/3	170
3	1	C3: 5′-GTAGAATTCAAGACCTTCCCAGGCTCTCCC-3′ C4: 5′-CTAGGAATTCTTAGGCCCCTCACCAAGGTG-3′	1.5	50/1	65/2	360
	2	C5: 5′-GTACAAGCTTTCAGCATTGACTCCCCACCT-3′ C6: 5′-GTAAAGCTTGGCCCCTGCTGGTTACCAGCT-3′	1.5	50/1	72/2	210
4/5	1	C7: 5′-CTAGAATTCCCTGTTTGTCTGGAAGCCCTC-3′ C8: 5′-ACACCGGCTGCAGGAGCCTG-3′	2.0	50/1	64/2	450
	2	C9: 5′-TGCTGGTGCCGCGCCCCAA-3′ C10: 5′-CTATTCGAAGCCGCCCGCCAGGATCCACCT-3′	2.0	50/1	64/2	330
6	1	C11: 5′-ATCGGCAGCTTCAGCTGCGA-3′ C12: 5′-CTAGGAATTCGCGGCACCAGCACCAGCTGC-3′	1.5	50/1	64/2	400
	2	C13: 5′-CTCCCTAGAAACCCTCCTGA-3′ C14: 5′-GTACAAGCTTGCGTGATTCCTGGGCGATGT-3′	1.5	50/1	64/2	210
7		C15: 5′-CTAGGAATTCCAGTCTCGGGAGGAGTGCCT-3′ C16: 5′-GTACAAGCTTTGCCAGGATGGACTCAGTGA-3′	1.5	55/1	72/2	260
8		C17: 5′-CTAGGAATTCCCTAAGCCTATGCCCATATG-3′ C18: 5′-ATCGAAGCTTCAAACCTGAACAGCCTGCCT-3′	2.5	63/2	72/3	310
9	1	C19: 5′-CTAGGAATTCCGCAGCCCACTCTGACTGTG-3′ C20: 5′-CTACAAGCTTGTTCTCAGACACCATGTTGC-3′	1.0	50/1	72/2	409
	2	C21: 5′-CTAGAATTCGGAGGCCAAGAGAAACCGCAC-3′ C22: 5′-CTATTCGAATCCATTGCCATGCAAAAGCCC-3′	1.5	50/1	72/2	400

and extension at 72°C for 1 minute. The presence of factor V and prothrombin was confirmed by the presence of the *Hind*III restriction site in the A allele carriers.

Results

In this study, FVL, prothrombin G20210A, and protein C mutations were investigated in 50 patients with venous thrombosis. In all, 3 of the 50 patients had the FVL mutation, 1 patient (2%) was homozygous and 2 patients (4%) were heterozygous for the mutation, and the remaining patients showed no evidence of FVL. One of the heterozygous patients was a 55-year-old woman with CVT. The second heterozygous patient was a 39-year-old woman with DVT. The homozygous patient was 45-year-old woman with CVT. All 3 patients were free of other recognized risk factors. In the control group, the mutated allele of the factor V gene was not observed. Compared with controls, the odds ratios (OR) were 5.6 (95% confidence interval [CI]-0.047-674) and 10.96 (95% CI-0.104-1158) for homozygosity and heterozygosity, respectively (Table 2).

The prothrombin G20210A mutation was detected in heterozygote form (GA) in 2 patients (4%) with CVT and in 2 healthy subjects. There was no statistically significant difference between the patients and the control group ($P > .01$). When compared with the controls, the OR for prothrombin G20210A heterozygosity was 0.48 (95% CI-0.065-3.52) in the patients (Table 2). One of the patients also carried the protein C mutation, and her protein C level was also very low (38%).

The SSCP analysis of the 9 exons of the protein C gene revealed aberrant electrophoretic patterns in exons 3, 4, 8, and 9 in 9 patients. Four of the mutations were in exon 3, 2 were in exon 4, 2 were in exon 8, and 1 was in exon 9. Three patients with protein C mutations had PE, 5 patients had CVT, and 1 patient had DVT. Seven of these patients had low plasma protein C levels, whereas 2 individuals exhibited borderline (73% and 80%) levels. Plasma protein C antigen levels were in the normal range in control subjects (70%-140%).

Discussion

In circulation, the predisposition to thrombosis depends on the balance between procoagulant and anticoagulant systems. Various genetic defects that affect any gene in these systems have been associated with an increased risk of venous thrombosis.[18]

Table 2. Frequency of FVL, Prothrombin G20210A, and Protein C Mutations

Genotype	Frequency in Control Subjects	Frequency in Thrombosis	Frequency in PE and DVT	Frequency in CVT	Odds Ratio in Thrombosis (95% CI)
FVL heterozygous	0/25	2/50, 4% (–1.43 to 9.43)	1/19, 5.3% (–4.77 to 15.3)	1/31, 3.2% (–2.99 to 9.44)	10.96 (0.104-1158)
FVL homozygous	0/25	1/50, 2% (–1.88 to 5.88)	0/19	1/31, 3.2% (–2.99 to 9.44)	5.62 (0.047-674)
Prothrombin G20210A heterozygous	2/25, 8% (–2.63 to 18.63)	2/50, 4% (–1.43 to 9.43)	0/19	2/31, 6.5% (–2.19 to 15.09)	0.48 (0.065-3.52)
Protein C mutation	0/25	9/50, 18% (7.35 to 28.64)	4/19, 21% (2.72-39.38)	5/31, 16.12% (3.18-29.07)	55.57 (1.669-1851)
Protein C mutation and prothrombin heterozygous	0/25	1/50, 2% (–1.88 to 5.88)	0/19	1/31, 3.2% (–2.99 to 9.44)	5.62 (0.047-674)

NOTES: CVT = cerebral vein thrombosis; FVL = factor V Leiden; DVT= deep vein thrombosis; CI = confidence interval; PE = pulmonary embolism.

In this study, we investigated FVL, prothrombin G20210A, and protein C mutations in patients with venous thrombosis.

It has been convincingly demonstrated that heterozygous carriers of the FVL mutation have an increased risk of venous thrombosis compared with noncarriers.[17,19,20] Most of these studies investigate the correlation between FVL and recurrence of thrombosis.[18,21,22] When we examined the patients with venous thrombosis, 2 (4%) of the 50 patients were heterozygous and 1 patient (2%) was homozygous for the FVL mutation. The proportion of carriers of FVL in the white population varies from 2% to 15%. In our study group, the incidence of FVL was not significantly different from that in the healthy population.

We investigated the mutations in the patients after the first attack. Our study group was composed of 3 venous thrombosis groups: CVT, DVT, and PE.

Ma et al[23] reported that 25% of patients with DVT carry an FVL mutation. In our DVT group, the frequency of FVL was not as high as reported. Only one of the 12 patients with DVT had a homozygote FVL mutation. The other 2 heterozygous patients had CVT. Margaglione et al[24] reported that the frequencies of FVL mutations and/or prothrombin G20210A mutations were significantly higher in patients with DVT and PE than in the control group. However, a series of studies reported a lower prevalence of resistance to activated protein C or FVL mutation in patients with isolated PE.[18,25] In 2 series of autopsies, Vandenbroucke et al[26] did not find an association between the FVL mutation and fatal PE in pathology materials from patients. We isolated 7 PE samples, and none had an FVL mutation. In our study, patients with PE or DVT did not exhibit a prevalence of FVL mutation that was different from the prevalence in the control group. As a result, in the CVT (6.4%), DVT (8.3%), and PE groups, the incidence of FVL was not significantly different from that in the control group.

Some investigators have reported the association of the prothrombin G20210A allele with venous thromboembolism.[2,27,28] The first study describing the G20210A prothrombin gene mutation reported a nearly 3-fold increased risk of venous thrombosis in mutation carriers.[4] We found that this mutation is not common in our study group, occurring in only 2 of the 50 cases (4%). In the Leiden Thrombophilia Study (LETS), the prothrombin gene mutation was demonstrated in 6.2% of symptomatic patients.[29] The prevalence of the mutation in our patients was similar (3.8% vs 6.2%). The prevalences of the prothrombin G20210A mutation in the healthy control subjects were 2.3% in the LETS and 2.6% in ours. These results indicate that although the G20210A mutation is common, it is not a risk factor for venous thrombosis.

In many family reports, an association between a thrombotic tendency and a protein C deficiency has been established.[18,20,30] We found mutations in 9 of the 50 (18%) patients. This mutation frequency is slightly higher than the frequencies reported for the protein C gene in other investigations on venous thrombosis. This difference may be due to the ethnic backgrounds.

Broekmans et al[31] have shown that protein C may lead to an appreciably increased risk for thrombosis when combined with some other moderate

abnormality. Leroy-Matheron[32] reported that in heterozygous patients with the FVL mutation, the first thrombotic event occurred later than in heterozygous patients with protein C or protein S deficiency. Our results are consistent with this report. Seven patients carrying a protein C mutation had their first episode before age 44 years, and 1 of them had his first episode very early (at age 19 years). These results indicate that protein C deficiency may contribute to the formation of venous thrombosis at an early age ($P < .001$).

Rosendaal[33] reported that in familial cases, carriers of protein C deficiency had high risk of thrombosis compared with relatives without deficiency. No familial cases were present among our patients. However, there was still a significant difference in the protein C mutation frequency between the venous thrombosis and control groups ($P = .024$; OR = 1.220; CI = 1.071-1.389).

Our results indicate that patients with protein C deficiency had a greater risk of thrombosis compared with patients carrying FVL or prothrombin G20210A mutation.

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