Endothelial cell protein C receptor gene 6936A/G polymorphism is associated with venous thromboembolism

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Abstract. Venous thromboembolism (VTE) is a common, yet complex disorder. Genetic factors have been suggested to play a role in disease development. We, therefore, conducted a case-control study to examine the potential association of the 6936A/G polymorphism in the endothelial cell protein C receptor (EPCR) gene with the occurrence of venous thromboembolism. We measured the plasma levels of soluble EPCR (sEPCR) in blood samples collected from 112 patients with VTE and 112 age- and gender-matched healthy donors using enzyme-linked immunosorbent assay (ELISA) and amplified the EPCR gene product by PCR. Gene product bands were sequenced to identify EPCR gene polymorphisms. We found that the 6936 AG and GG genotypes were over-represented in the VTE patients. By multivariate analysis, subjects carrying the 6936 G allele were found to have an increased risk of thrombosis (OR=1.784; 95% CI, 1.113-2.891; P<0.05). In conclusion, the EPCR gene 6936A/G polymorphism, which is associated with elevated plasma sEPCR levels, is a potential candidate risk factor for venous thromboembolism.

Introduction

In the past 50 years, the molecular bases of blood coagulation and the anticoagulant pathways have been explained, and several genetic risk factors for venous thrombosis have been identified. These genetic risk factors affect the natural anticoagulant mechanisms and result in a hypercoagulable state due to an imbalance between procoagulant and anticoagulant forces. Thrombosis is a lifelong risk and thrombotic events tend to occur when one or more of the circumstantial risk factors come into play. Venous thromboembolism (VTE) is a typical multifactorial disease whose pathogenesis involves acquired and genetic mechanisms.

Acquired factors involve prolonged bedrest, surgery, pregnancy and malignancies. The genetic factors include mutations in factors involved in the coagulation-fibrinolytic system, such as mutations in Factor V Leiden (1), prothrombin G20210A (2,3) or methylenetetrahydrofolate reductase C677T (4), and mutations resulting in deficiency of antithrombin, protein C (PC) or protein S. Evidence indicates that the PC pathway is part of the natural anticoagulation system and plays an important role in maintaining the balance between coagulation and anticoagulation. Previous studies showed that the endothelial protein C receptor (EPCR), a key component of the PC pathway (5), can increase the activation efficiency of PC 5-fold, leading to markedly elevated anticoagulation activity. The most recent study has shown that blocking EPCR can accelerate thrombus development *in vivo* (6). These findings indicate that EPCR may play a role in the susceptibility to and the development of venous thrombosis. To elucidate this possibility more thoroughly, we performed a case-control study to investigate the role of EPCR in VTE and to examine the relationship between the presence of the 6936A/G polymorphism of EPCR and the occurrence of VTE.

Materials and methods

Study subjects. Between January 2008 and June 2010, 112 patients (64 males, 48 females) who had been diagnosed by duplex ultrasonography with lower extremity deep venous thrombosis (DVT) at Shandong Provincial Hospital, Shandong University, China, were recruited into the VTE group. The mean age was 46.8±9.1 years (range, 27-92 years). Of the patients, 59 (52.7%) were outpatients and 53 (47.3%) were hospitalized patients. Patients with hematological diseases, liver and kidney dysfunctions, infections, autoimmune diseases, tumors, or those receiving thrombolytic treatment or anticoagulant treatment were excluded from this study. Venous thrombosis was localized on the left side in 67 patients (59.8%), on the right side in 39 patients (34.8%) and on both sides in five patients (4.5%). A total of six patients had pulmonary embolism (PE) (5.4%), five patients presented with DVT and PE and one patient had an idiopathic PE. The characteristics of the patients are shown in Table I. A total of 112 healthy

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Table I. Patient characteristics.

Characteristic	n	%
Left lower extremity DVT	67	59.8
Right lower extremity DVT	39	34.9
Thrombosis on both sides	5	4.5
DVT at iliofemoral level	30	26.8
DVT at femoropopliteal level	27	24.1
DVT at calf level	54	48.2
Idiopathic PE	1	0.9
PE secondary to DVT	5	4.5

DVT, deep venous thrombosis; PE, pulmonary embolism.

unrelated subjects were recruited into the control group after being interviewed regarding whether they had been diagnosed with VTE or other associated diseases. The mean age of the normal donors was 48.5±7.4 years (range, 19-81 years). In total, 71 (63.4%) of the healthy subjects were males and 41 (36.6%) were females. Characteristics of the study population are shown in Table II. Informed consent was obtained from all study subjects following explanation of the nature of the study. The study was approved by Shandong University Research Ethics Committee, China.

DNA extraction and genotyping. Venous blood was obtained from each subject and genomic DNA was extracted using a DNA extraction kit (Tianamp Biotech, Beijing, China) according to the manufacturer's instructions, then stored at -70˚C until use. ELISA was applied to detect levels of plasma sEPCR in patient plasma samples (USCNLIFE, Beijing, China). Genomic DNA was analyzed by polymerase chain reaction (PCR). The primers for EPCR were designed as previously reported with the following sequences for sense, 5'-GCTTCAGTCAGTTGGTAAAC-3' and antisense, 5'-TCTGGCTTCACAGTGAGCTG-3', which were used in a 25-μl mixture for amplification. The cycling conditions for PCR were 30 cycles of denaturation (94˚C for 45 sec), annealing (57˚C for 45 sec) and extension (72˚C for 45 sec). A preheating step at 94˚C for 5 min and a final extension step for 7 min at 72˚C were also performed. The products were stored at 4˚C. Amplified products were later mixed and buffered with restriction endonuclease *Pst*I and sustained in a water bath overnight for digestion. Finally, all products were observed following the polyacrylamide gel electrophoresis.

Statistical approach. All of the statistical analyses were carried out using SPSS 13.0 statistical software (SPSS Inc., Chicago, IL, USA) and data were presented as the mean ± standard deviation. Comparisons between two groups were performed by the independent t-test; Chi-square analysis was applied to determine the difference in the genotype and gene frequency. Genotype and the risk for VTE were expressed by odds ratio (OR) at a 95% confidence interval (CI). A value of P<0.05 was considered to indicate statistical significance.

Table II. Characteristics of the case-control study population.

	Total	Males	Females	Mean age (years)
Cases	112	64	48	46.8±9.1
Controls	112	71	41	48.5±7.4

Characteristics of the case-control study population show that the VTE group and the control group are age- and gender-matched (P>0.05).

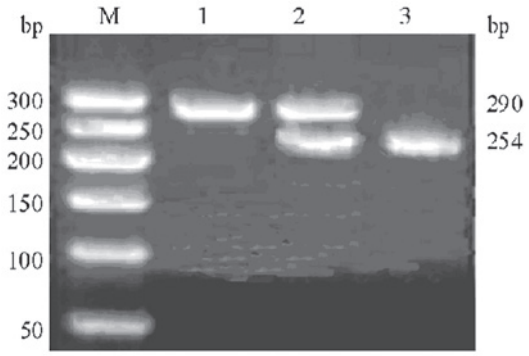

Figure 1. EPCR gene 6936A/G enzyme cut electrophoresis of restriction endonuclease *Pst*I. M, DNA molecular weight marker; lane 1, GG genotype; lane 2, AG genotype; lane 3, AA genotype.

Results

Three genotypes, AA, AG and GG, in the EPCR gene at position 6936 were noted in the VTE group as well as in the control group. Fig. 1 shows the EPCR gene 6936A/G enzyme cut electrophoresis of restriction endonuclease *Pst*I. The frequencies of EPCR 6936A/G genotypes and alleles in the VTE and healthy subjects are shown in Table III. In the VTE group, the frequency of mutational genotypes (AG+GG) was 36.6% (OR=1.912; 95% CI, 1.064-2.818), which was significantly higher than that in the control group (20.5%, P<0.05). Furthermore, the frequency of the G allele (19.6%; OR=1.784; 95% CI, 1.113-2.891; P<0.05) in the VTE group was significantly higher than that in the control group (10.8%).

The plasma level of sEPCR in the VTE group (132.6±61.3 ng/ml) was significantly higher than that in the control group (94.1±30.6 ng/ml; P<0.05). Levels of sEPCR among different genotypes are shown in Fig. 2. In the VTE group, the level of sEPCR in subjects with the AG genotype and GG genotype as a whole (189.2±53.7 ng/ml) was significantly higher than in subjects with the AA genotype (92.1±25.0 ng/ml, P<0.05). In the control group, the level of sEPCR in subjects with the AG genotype and GG genotype as a whole (143.5±54.3 ng/ml) was significantly higher than that in subjects with the AA genotype (76.3±26.8 ng/ml, P<0.05).

Discussion

VTE is increasingly regarded as a polygenic disease. It was reported that approximately 60% of VTE patients have genetic

Table III. Frequencies of genotypes and alleles.

	Genotype frequency n (%)			Allele frequency	
	AA n (%)	AG n (%)	GG n (%)	A n (%)	G n (%)
VTE group	69 (61.6)	38 (33.9)	3 (2.7)	180 (80.4)	44 (19.6)
Control group	89 (79.5)	22 (19.6)	1 (0.9)	200 (89.2)	24 (10.8)

In the VTE group, frequency of mutational genotypes (AG+GG) was 36.6%, which was significantly higher than that in the control group (20.5%, P<0.05). Meanwhile, the frequency of the G allele (19.6%, P<0.05) in the VTE group was significantly higher than that in the control group (10.8%).

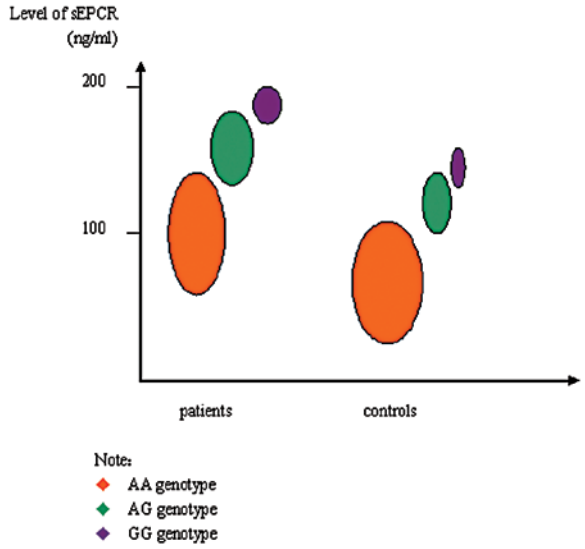

Figure 2. The plasma level of sEPCR in the VTE group was significantly higher than that in the control group (132.6±61.3 vs. 94.1±30.6 ng/ml, P<0.05). In the two groups, the level of sEPCR in subjects with the AG or GG genotypes was significantly higher than that in the subjects with the AA genotype.

risk factors and more than one third of VTE patients have a family history (7). Genetic risk factor assessment has become an integral component of the diagnostic evaluation of patients who present with the signs and symptoms of venous thrombosis (8). Thrombophilia is caused by a set of acquired and inherited conditions that confer a tendency for thrombus formation. The PC pathway is a part of the natural anticoagulation system and plays an important role in maintaining the balance between coagulation and anticoagulation. PC, a vitamin K-dependent zymogen, is activated at the endothelial surface when thrombin binds to thrombomodulin, a protein that transforms the procoagulant enzyme into a potent activator of PC. In the presence of its cofactor, protein S, activated protein C (aPC) inactivates factors Va and VIIIa, thereby reducing thrombin generation. EPCR, a type 1 transmembrane protein (9) that is homologous to the major histocompatibility complex class 1/CDI family of proteins, was identified more recently at the surface of endothelial cells. EPCR demonstrates a relatively endothelial cell-specific expression pattern, with the expression levels being notably higher on large vessel endothelium, particularly large arteries, and low or absent on capillaries. This receptor, which binds PC or aPC with the same affinity, is mainly expressed on endothelial cells of large vessels. The EPCR concentration plays a major role in determining protein C activation. Up-regulation of EPCR by thrombin (10) or down-regulation of EPCR expression by cytokines or proteolytic attack (11) would, based on the present study, contribute directly to PC activation and serve to modulate the critical control of the blood clotting process.

The mechanism relating elevated sEPCR levels to venous thrombosis remains to be determined. Increased sEPCR concentrations result in decreased aPC generation and inhibit generated aPC, with possible implications for the regulation of coagulation, since a low circulating aPC level has been shown to be a risk factor for venous thromboembolism (11). Extensive analyses of the EPCR gene identified several polymorphisms, which involved 3 haplotypes (13,14). The A3 haplotype, one of the three, was markedly associated with high sEPCR levels. The high constitutive sEPCR levels observed in A3 haplotype carriers may reduce the efficiency of the PC system and predispose these subjects to venous thrombosis. The molecular mechanism by which the A3 haplotype increases the plasma sEPCR level remains to be identified. Several polymorphisms defining the A3 haplotype are located within intronic regions. One of the possible explanations for the increased sEPCR levels associated with the A3 haplotype is a putative conformational change in the protein due to the Ser 219 to Gly substitution (resulting from the 6936 A to G mutation). This residue is located in the transmembrane (15) domain, near another glycine residue, and these two adjacent Gly residues may destabilize the helical transmembrane domain and thus may change the exposure of the cleavage site, resulting in a protein that is more sensitive to metalloproteinase cleavage.

In our study, the frequencies of the 6936AG and 6936GG genotypes in the VTE group were significantly higher than that in the control group, which suggest an increased risk for thrombosis in patients with the 6936AG and 6936GG genotypes. In our study, the G allele frequency in the VTE patients was increased when compared with the healthy control subjects, which was consistent with other studies (16,17). This result suggests that the EPCR gene indicates susceptibility to VTE and is associated with VTE pathogenesis. In conclusion,

our results indicate that the plasma sEPCR level is associated with the 6936A/G polymorphism of the EPCR gene. The plasma sEPCR level in VTE patients was higher than that in the healthy control subjects and the frequencies of the EPCR gene 6936AG and 6936GG genotypes in the VTE patients were markedly increased when compared with the healthy subjects. These findings suggest that the EPCR gene 6936A/G polymorphism may be a candidate risk factor for VTE.

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