

Platelet endothelial cell adhesion molecule-1 gene 125C/G polymorphism is associated with deep vein thrombosis

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Abstract. Deep vein thrombosis (DVT) is a common disorder that is associated with high morbidity and mortality. Genetic factors have been suggested to influence the predisposition towards thrombosis and the incidence of DVT. Platelet endothelial cell adhesion molecule-1 (PECAM-1) is a key adhesion molecule that is involved in platelet function and maintenance of endothelial cell junctions. To date, no studies have examined the association between polymorphisms in PECAM-1 and DVT. The present study analyzed the single nucleotide polymorphisms (SNPs) of PECAM-1, namely Leu125Val (C373G), Asn563Ser (T1688C) and Gly670Arg (C2008T), in Chinese patients with DVT and age- and gender-matched controls, using polymerase chain reaction-restriction fragment length polymorphism analysis. Furthermore, plasma soluble PECAM-1 (sPECAM-1) levels were quantified by ELISA. The results of the present study demonstrated significantly higher genotype and allele frequencies of the Leu125Val polymorphism in PECAM-1 in the DVT group as compared with those in the control group ($P < 0.05$). The plasma levels of sPECAM-1 in the DVT group (83.4 ± 23.5 ng/ml) were also significantly higher as compared with those in the control group (60.4 ± 19.4 ng/ml, $P < 0.01$). In the patients with DVT, plasma levels of sPECAM-1 were significantly higher in those with the Leu/Val and Val/Val genotypes as compared with those possessing the Leu/Leu genotype ($P < 0.05$). The PECAM-1 Leu125Val polymorphism was shown to be associated with an increased risk of DVT

and PECAM-1 protein expression levels in venous vessels. In patients with DVT, the PECAM-1 Leu/Val and Val/Val genotypes were associated with delayed thrombus resolution, as determined by thrombus scoring, as compared with that in patients possessing the Leu/Val genotype. In conclusion, the present study indicated that PECAM-1 Leu125Val polymorphism and sPECAM-1 levels may be associated with DVT.

Introduction

Venous thromboembolism (VTE), which includes deep vein thrombosis (DVT) and pulmonary embolism (PE), is a common disorder with a high annual incidence of ~131.5 cases per 100,000 people in the general population (1). DVT is characterized by the formation of an occlusive blood clot in the venous vascular system, which can potentially result in PE, due to detachment and embolization of thrombi into the lung. Therefore, DVT and PE are regarded as two different stages of the same disease, and are associated with common risk factors caused by genetic, environmental and behavioral interactions (2,3). DVT is a major cause of cardiovascular-associated mortality and its treatment is limited to palliation, including mechanical leg compression and anticoagulation therapy (4).

The underlying cellular mechanisms of DVT have remained elusive. Slow blood flow, abnormal blood components and vein endothelial injury are considered to be the three classical factors that participate in the pathogenesis of DVT (5). Reduced blood flow leads to adherence of circulating neutrophils and monocytes to the activated endothelium within hours, thus activating platelets, circulating tissue factor and factor XII, resulting in the initiation and propagation of DVT (6). Platelets perform surveillance on vascular injury and are capable of vascular repair and hemostasis by quick adhesion and aggregation at the site of injury. However, disruption of hemostasis may lead to abnormally enhanced platelet adhesion and aggregation, which can cause thrombotic disorders such as DVT (7). Out of all of these processes, platelet-endothelial adhesion has a key role in the initiation and promotion of DVT. von Willebrand factor (VWF) was previously shown to be required for thrombus formation in a murine model of DVT through promoting platelet adhesion and the subsequent adherence of platelets to

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endothelial cells (8). Furthermore, platelets can communicate with monocytes and neutrophils to initiate and propagate venous thrombosis in mice with DVT by promoting leukocyte recruitment and neutrophil-dependent coagulation (9). The mechanisms underlying platelet-endothelial adhesion and DVT remain to be fully elucidated; however, the process is known to be mediated by various cell adhesion molecules, including platelet endothelial cell adhesion molecule-1 (PECAM-1) (10).

PECAM-1 is a 130-kDa cell surface glycoprotein that belongs to the immunoglobulin gene superfamily of cell adhesion molecules. PECAM-1 is expressed on the surface of circulating platelets, monocytes, neutrophils and endothelial cell intercellular junctions (11). PECAM-1 is involved in inhibition of platelet function (12) and maintenance of endothelial barrier integrity (13), both of which are major determinants of venous thrombosis. Furthermore, PECAM-1-deficient mice with DVT exhibited larger thrombi over longer periods of time. In addition, higher plasma levels of sPECAM-1 were detected in patients with delayed resolution of thrombi, as compared with patients whose thrombi resolved normally (14).

The PECAM-1 gene is located on human chromosome 17q23 and consists of 16 exons. A number of single nucleotide polymorphisms (SNPs) have been identified in PECAM-1 (15). Out of the 11 SNPs in human PECAM-1, only three have been shown to be associated with disease: Leu125Val (C373 G), Asn563Ser (T1688C) and Gly670Arg (C2008T) (16). The Leu125Val polymorphism is located in exon 3 and consists of a leucine to valine alteration, Asn563Ser is located in exon 8 and consists of an asparagine to serine alteration, and Gly670Arg is located in exon 12 and consists of a glycine to arginine alteration. PECAM-1 gene polymorphisms have previously been shown to be associated with atherosclerosis and myocardial infarction (17,18). However, no studies have yet examined the association between PECAM-1 polymorphisms and DVT. Furthermore, plasma sPECAM-1 levels were previously shown to be increased in DVT patients with delayed thrombus resolution; however, the association between plasma sPECAM-1 levels and PECAM-1 polymorphisms in DVT remains elusive.

The present study investigated the frequency of PECAM-1 Leu125Val, Asn563Ser, and Gly670Arg polymorphisms in patients with DVT as compared with those in healthy controls. Furthermore, the association between these polymorphisms and plasma sPECAM-1 levels, vascular PECAM-1 expression, and thrombus burden was evaluated in the patients with DVT.

Materials and methods

Subjects. The present study consisted of 115 patients with DVT (67 males and 48 females, between 30 and 83 years of age). All of the patients were diagnosed with lower extremity DVT by duplex ultrasonography between September 2011 and March 2013, at Shandong Provincial Hospital affiliated to Shandong University (Jinan, China) and were recruited into the DVT group. The exclusion criteria were as follows: Hematological diseases, liver or kidney dysfunction, tumors, infections, autoimmune diseases, or coexisting symptomatic pulmonary embolism. The DVT group consisted of 63 patients with left lateral DVT (54.8%), 35 patients with right lateral DVT (30.4%) and 17 patients with bilateral DVT (14.8%). A total of 104 healthy unrelated subjects, both age- and gender-matched, were recruited into the control

group (60 males and 44 females, between 29 and 79 years of age). The control subjects underwent a routine medical check-up and none of them were diagnosed with DVT or other associated diseases. Written informed consent, in accordance with the Declaration of Helsinki, was obtained from all of the study subjects, and the study was approved by the Shandong University Research Ethics Committee (Jinan, China).

Determination of PECAM-1 genotype. Peripheral venous blood was obtained from all of the patients with DVT and the healthy controls, and was promptly centrifuged at 1,000 x g for 10 min. Genomic DNA was extracted from the leucocytes using a DNA Extraction kit (Qiagen, Manchester, UK), according to the manufacturer's instructions. The extracted DNA was then stored at -70°C until further use. PECAM-1 Leu125Val (C373G), Asn563Ser (T1688C) and Gly670Arg (C2008T) genotype frequencies were determined by polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) analysis. The PCR primers were designed and synthesized based on the GenBank reference sequence (accession no. NC_000017), as previously reported (19), with the following sequences: Leu125Val sense, 5'-GCTCCATCTGCTTGCTGT-3' and anti-sense, 5'-TGTCAGCACCACCTCTCACG-3'; Asn563Ser sense, 5'-TGGAGACCCTGACTCACCTC-3' and anti-sense, 5'-TGCAATGTGCTGTGAATGAA-3'; and Gly670Arg sense, 5'-TGGGAAATTATCCACAGTCCTTCA-3' and antisense, 5'-CAACTAGGTCACAATGACGATGCC-3' by Sangon Biotech Co., Ltd. (Shanghai, China). The PCR amplification was performed in a total volume of 25 µl using the PCR Amplification kit (Takara Biotechnology Co., Ltd., Dalian, China) on PCR amplification reaction apparatus (Tgradient; Biometra, Göttingen, Germany). The cycling conditions for PCR were set as follows: Initiation at 94°C for 5 min, followed by 30 cycles of denaturation at 94°C for 40 sec, annealing at 60°C for 4 sec and extension at 72°C for 10 sec, with a final extension step at 72°C for 7 min. The amplified PCR products were then incubated with restriction endonuclease *PvuII* (Leu125Val), *NheI* (Asn563Ser) or *MspI* (Gly670Arg) (Takara Biotechnology Co., Ltd.) overnight for digestion. All of the PCR products were subsequently separated by 8% polyacrylamide gel electrophoresis. The molecular weight marker (GM303) was obtained from BBI Life Sciences Corporation (Shanghai, China).

Determination of sPECAM-1 levels. Plasma was collected from the venous blood samples of the patients with DVT within 24 h of diagnosis by centrifugation at 1,000 x g for 20 min. The plasma samples were then stored in aliquots at -70°C until further use. Measurements of sPECAM-1 levels were performed using the Human sPECAM-1 ELISA kit (Bender MedSystems GmbH, Vienna, Austria), according to the manufacturer's instructions. The color density of the samples was measured at a wavelength of 450 nm using an ELISA plate reader (Ricso RK201; Shenzhen Ricso Technology Co., Ltd, Shenzhen, China). A standard curve was constructed using the standards supplied in the ELISA kit (Range, 0-1000 ng/ml), in order to determine the concentrations of sPECAM-1.

Western blot analysis. Human samples of venous vessel walls were obtained from patients with DVT during variceal surgeries (n=6). Venous vessel walls were harvested and

were immediately snap frozen in liquid nitrogen. Prior to the experiment, the venous vessel walls were lysed by RIPA buffer [50 mM Tris-HCl (pH 7.5), 1% Nonidet P-40, 0.25% Na-deoxycholate, 150 mM NaCl, 1 mM EDTA and complete protease inhibitor cocktail (Roche, Indianapolis, IN, USA)]. Following centrifugation at 20,000 x g and 4°C for 15 min, the supernatant was collected. The protein concentrations were determined using a Bicinchoninic Acid Protein Concentration Assay kit (Beijing Biosea Biotechnology Co. Ltd., Beijing, China). Equal amounts of protein (50 µg) were separated by 10% SDS-PAGE and electrophoretically transferred to a polyvinylidene fluoride (PVDF) membrane (Bio-Rad Laboratories, Inc., Hercules, CA, USA). The membrane was then blocked with 3% bovine serum albumin (Sigma-Aldrich, St. Louis, MO, USA) in Tris-buffered saline containing 0.05% Tween® 20 (Sigma-Aldrich) for 1 h, followed by incubation with a primary mouse monoclonal antibody targeting human PECAM-1 (1:1,000 dilution; Santa Cruz Biotechnology, Inc., Dallas, TX, USA) at 37°C overnight. The PVDF membrane was then incubated with horseradish peroxidase-conjugated rabbit anti-mouse polyclonal secondary antibody (1:1,000 dilution; Santa Cruz Biotechnology, Inc.) for 2 h at room temperature. An Enhanced Chemiluminescence substrate (Pierce ECL Plus Western Blotting substrate; Pierce Biotechnology, Inc., Rockford, IL, USA) was used to visualize the density of the targeted bands. β-actin was used as an internal control.

Determination of thrombus burden. The thrombus score reflects the thrombus burden in the leg vein and was calculated in the present study by manual complete compression ultrasonography using ProSound Alpha 7 Doppler ultrasonography (Hitachi-Aloka Medical Ltd., Tokyo, Japan) at 75 MHz (20). The score for each affected venous segment was calculated from thrombus diameter under full compressed conditions, relative to the diameter of the corresponding artery. When the compressed thrombus diameter was >1.5 times the arterial diameter, the segment score was increased 1.5-fold (14). When the compressed thrombus diameter was <0.5 times the arterial diameter, the segment score was reduced 0.5-fold. The thrombus score was calculated as the sum of each segment: External iliac vein, 8; common femoral vein, 4; proximal superficial femoral vein, 4; distal superficial femoral vein, 3; deep femoral vein, 2; popliteal vein, 2; peroneal veins, 2; and posterior tibial veins, 2. A baseline score >4 was an inclusion criterion for the DVT group in the present study. Follow-up ultrasonography was performed at 28 days after diagnosis in each of the patients with DVT, and a duplex scan was performed to evaluate residual vein thrombus as well as to calculate the change in the thrombus score (Δ thrombus score = baseline thrombus score - thrombus score on day 28). A Δ thrombus score <4 was defined as delayed thrombus resolution. All of the patients with DVT received compression stockings and oral anticoagulation (warfarin) therapy (Coumadin®, Bristol-Myers Squibb, New York City, NY, USA).

Statistical analysis. All quantitative data are expressed as the mean \pm standard deviation. The commercially available software SPSS version 14.0 (SPSS Inc., Chicago, IL, USA) was used for all statistical analyses. An independent t-test was used to compare results between two groups. A χ^2 analysis was used

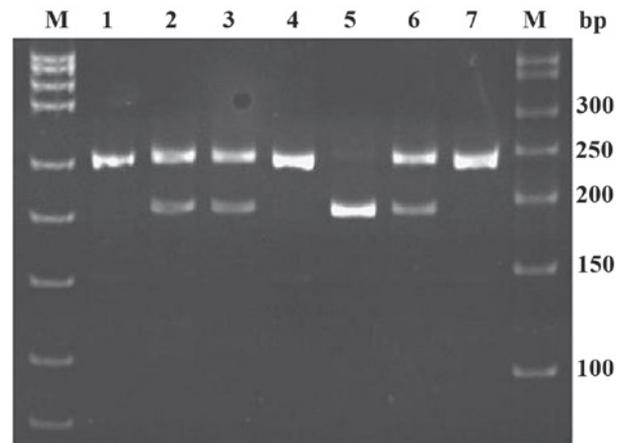


Figure 1. PECAM-1 Leu125Val (C373G) polymorphism was analyzed by enzyme-cut electrophoresis with restriction endonuclease *PvuII*. M, DNA molecular weight marker; lanes 1, 4 and 7, 373 CC (125 Leu/Leu) homozygous genotype, 245 bp; lanes 2, 3 and 6, 373 CG (125 Leu/Val) heterozygous genotype, 245 bp and 193 bp; lane 5, 373 GG (125 Val/Val) homozygous genotype, 193 bp. PECAM-1, platelet endothelial cell adhesion molecule-1.

to determine the differences in genotype and allele frequencies. Unconditional logistic regression models were used to determine odds ratios (ORs) and 95% confidence intervals [95% confidence intervals (CIs)]. The Hardy-Weinberg equilibrium test was performed on the control and DVT groups to determine whether the samples were a typical representative groups of the whole population. $P < 0.05$ was considered to indicate a statistically significant difference.

Results

Val/Val genotype of PECAM-1 is associated with an increased risk of DVT. No statistically significant differences were observed between the age and gender of the control and DVT groups, thus indicating that the two groups were matched for age as well as gender. The plasma sPECAM-1 levels were significantly higher in the DVT group (83.4 \pm 23.5 ng/ml), as compared with those in the control group (60.4 \pm 19.4 ng/ml) ($P < 0.05$; Table I). PECAM-1 genotype and allele frequencies of the Leu125Val, Asn563Ser and Gly670Arg polymorphisms were evaluated in the patients with DVT and the healthy controls. There were significant differences in the genotype and allele frequencies of the PECAM-1 Leu125Val polymorphism between the control and DVT groups ($P < 0.01$). The frequencies of Leu/Leu, Leu/Val and Val/Val genotypes of the Leu125Val polymorphism were 24.0, 53.9 and 22.1% in controls, and 12.2, 47.8 and 40.0% in the patients with DVT, respectively (Table II). However, there were no significant differences in the genotype and allele frequencies of the Asn563Ser and Gly670Arg polymorphisms between the control and DVT groups. The genotype distributions of the three polymorphisms were in Hardy-Weinberg equilibrium among the control and DVT groups (Table III). A PCR-RFLP assay was used to analyze the Leu125Val polymorphisms of PECAM-1, and the following fragments were detected: One 245 bp DNA fragment in CC homozygous subjects, three DNA fragments of 52, 193 and 245 bp in CG heterozygous subjects and two DNA fragments of 52 and 193 bp in GG homozygous subjects (Fig. 1). The 52-bp fragment was not visible in the electrophoresis gel

Table I. Characteristics of the patients with DVT and the healthy controls.

Characteristic	Controls (n=104) n	DVT (n=115) n	P-value
Age (years)	51.6±9.2	53.8±9.5	0.078
Gender (male/female)	60/44	67/48	0.11
sPECAM-1 (ng/ml)	60.4±19.4	83.4±23.5	<0.01

The DVT and control groups were matched for both age and gender. Plasma levels of sPECAM-1 in the DVT group were significantly higher as compared with those in the control group ($P<0.01$). DVT, deep vein thrombosis; sPECAM-1, soluble platelet endothelial cell adhesion molecule-1.

Table II. Genotype and allele frequencies of PECAM-1 polymorphisms in patients with DVT and healthy controls.

Polymorphism	Controls (n=104) n (%)	DVT (n=115) n (%)	χ^2	P-value
Leu125Val genotypes			10.25	<0.01
Leu/Leu	25 (24.0)	14 (12.2)		
Leu/Val	56 (53.9)	55 (47.8)		
Val/Val	23 (22.1)	46 (40.0)		
Alleles			9.85	<0.01
Leu	106 (51.0)	83 (36.1)		
Val	102 (49.0)	147 (63.9)		
Asn563Ser genotypes			3.94	>0.05
Asn/Asn	27 (26.0)	24 (20.9)		
Asn/Ser	55 (52.9)	53 (46.1)		
Ser/Ser	22 (21.1)	38 (33.0)		
Alleles			3.16	>0.05
Asn	109 (52.4)	101 (43.9)		
Ser	99 (47.6)	129 (56.1)		
Gly670Arg genotypes			2.47	>0.05
Gly/Gly	26 (25.0)	22 (19.1)		
Gly/Arg	53 (51.0)	58 (50.4)		
Arg/Arg	25 (24.0)	35 (30.5)		
Alleles			1.65	>0.05
Gly	105 (50.5)	102 (44.3)		
Arg	103 (49.5)	128 (55.7)		

Significant differences were present among the genotype and allele frequencies of Leu125Val between the control and DVT groups ($P<0.01$). There were no significant differences in the frequencies of Asn563Ser or Gly670Arg between the control and DVT groups ($P<0.05$). DVT, deep vein thrombosis; PECAM-1, platelet endothelial cell adhesion molecule-1.

due to its small size. The Leu/Val and Val/Val genotypes were associated with a significantly increased risk of DVT, as compared with the Leu/Leu genotype ($P<0.01$) (Table IV).

Association between sPECAM-1 levels and PECAM-1 Leu125Val polymorphism. An ELISA was conducted to detect the plasma sPECAM-1 levels in the control and DVT groups. Significantly higher sPECAM-1 levels were observed in the patients with DVT, as compared with those in the healthy controls (Table I). The sPECAM-1 levels were further analyzed in both groups with regards to the three Leu125Val

genotypes of PECAM-1. sPECAM-1 levels were significantly associated with the Leu125Val polymorphism in the control as well as in the DVT group. The plasma sPECAM-1 levels were significantly higher in DVT patients with the homozygous Val/Val genotype (95.2 ± 22.4 ng/ml, $n=46$) or the heterozygous Leu/Val genotype (78.7 ± 20.9 ng/ml, $n=55$), as compared with those in the patients with the homozygous Leu/Leu genotype (62.8 ± 15.9 ng/ml, $n=14$, $P<0.01$, respectively). Furthermore, significantly higher plasma sPECAM-1 levels were detected in the subjects with the Val/Val genotype as compared with those in the Leu/Val genotype (Fig. 2).

Table III. PECAM-1 genotype distribution of Leu125Val, Asn563Ser and Gly670Arg in Hardy-Weinberg equilibrium.

Group	Genotype	Observed value n (%)	Predicted value n (%)	χ^2	P-value
Leu125Val controls	Leu/Leu	25 (24.0)	27 (26.0)	0.31	>0.05
	Leu/Val	56 (53.9)	52 (50.0)		
	Val/Val	23 (22.1)	25 (24.0)		
DVT	Leu/Leu	14 (12.2)	15 (13.0)	0.08	>0.05
	Leu/Val	55 (47.8)	53 (46.1)		
	Val/Val	46 (40.0)	47 (40.9)		
Asn563Ser controls	Asn/Asn	27 (26.0)	28 (26.9)	0.19	>0.05
	Asn/Ser	55 (52.9)	52 (50.0)		
	Ser/Ser	22 (21.1)	24 (23.1)		
DVT	Asn/Asn	24 (20.9)	22 (19.1)	0.29	>0.05
	Asn/Ser	53 (46.1)	57 (49.6)		
	Ser/Ser	38 (33.0)	36 (31.3)		
Gly670Arg controls	Gly/Gly	26 (25.0)	27 (26.0)	0.03	>0.05
	Gly/Arg	53 (51.0)	52 (50.0)		
	Arg/Arg	25 (24.0)	25 (24.0)		
DVT	Gly/Gly	22 (19.1)	23 (20.0)	0.03	>0.05
	Gly/Arg	58 (50.4)	57 (49.6)		
	Arg/Arg	35 (30.5)	35 (30.4)		

Data were tested in the control and DVT groups. DVT, deep vein thrombosis; PECAM-1, platelet endothelial cell adhesion molecule-1.

Table IV. PECAM-1 polymorphisms and risk of DVT.

Genotype	Controls (n=104) n (%)	DVT (n=115) n (%)	OR (95% CI)	P-value
Leu/Leu	25 (24.0)	14 (12.2)	1	
Leu/Val	56 (53.9)	55 (47.8)	1.75 (1.22-2.53)	<0.01
Val/Val	23 (22.1)	46 (40.0)	3.57 (2.47-5.16)	<0.01
Leu/Val+Val/Val	79 (76.0)	101 (87.8)	2.28 (1.58-3.29)	<0.01

Leu/Val and Val/Val genotypes were significantly correlated with an increased risk of DVT, as compared with the Leu/Leu genotype. PECAM-1, platelet endothelial cell adhesion molecule-1; DVT, deep vein thrombosis; OR, odds ratio; CI, confidence interval.

Protein expression levels of PECAM-1 in the three Leu125Val genotypes of PECAM-1. Western blot analysis was performed to detect the PECAM-1 protein expression levels in the venous vessels of the patients with DVT. PECAM-1 protein expression levels were significantly lower in the patients with the Val/Val genotype, as compared with those with the Leu/Leu and Leu/Val genotypes (P<0.01, respectively). Furthermore, significantly lower PECAM-1 protein expression levels were detected in the patients with the Leu/Val genotype, as

compared with those with the Leu/Leu genotype (P<0.05; Fig. 3A and B).

Patients with Leu/Val and Val/Val genotypes have a higher thrombus burden and delayed thrombus resolution. Complete compression ultrasonography was performed to evaluate the thrombus burden in each patient with DVT at the time of diagnosis and 28 days thereafter. The baseline thrombus score was significantly higher in patients with

Table V. PECAM-1 polymorphisms and thrombus resolution in DVT.

Genotype	Leu/Leu	Leu/Val	Val/Val
Mean thrombus score baseline (\pm SD)	14.1 (\pm 2.5)	15.5 (\pm 2.8)	16.9 (\pm 2.9) ^{a,b}
Mean thrombus score d 28 (\pm SD)	9.3 (\pm 2.2)	11.9 (\pm 2.5) ^a	13.7 (\pm 2.3) ^{a,b}
Mean Δ thrombus score (\pm SD)	4.8 (\pm 1.3)	3.6 (\pm 1.5) ^a	3.3 (\pm 1.7) ^a
Normal thrombus resolution	11	27	17
Delayed thrombus resolution	5	30	25

Patients with Leu/Val and Val/Val genotypes had delayed thrombus resolution. Normal thrombus resolution: Δ thrombus score \geq 4. Delayed thrombus resolution: Δ thrombus score $<$ 4. SD, standard deviation. ^aP $<$ 0.05, as compared with the Leu/Leu genotype. ^bP $<$ 0.05, as compared with the Leu/Val genotype. DVT, deep vein thrombosis.

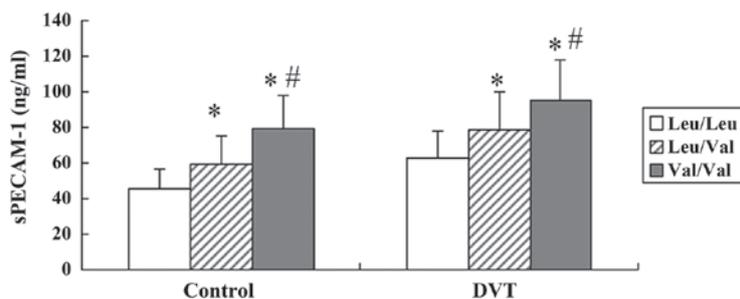


Figure 2. Association between the levels of sPECAM-1 and the PECAM-1 Leu125Val polymorphism in the control and DVT groups. Plasma sPECAM-1 levels were significantly higher in the subjects with the Val/Val genotype, as compared with those possessing the Leu/Leu and Leu/Val genotypes, in both the control and DVT groups. There was also a significant difference in the plasma sPECAM-1 levels between the subjects with the Leu/Leu and Leu/Val genotypes. Data is presented as the mean \pm standard deviation. *P $<$ 0.01, as compared with the Leu/Leu genotype. #P $<$ 0.01, as compared with the Leu/Val genotype. DVT, deep vein thrombosis; PECAM-1, platelet endothelial cell adhesion molecule-1; sPECAM-1, soluble PECAM-1.

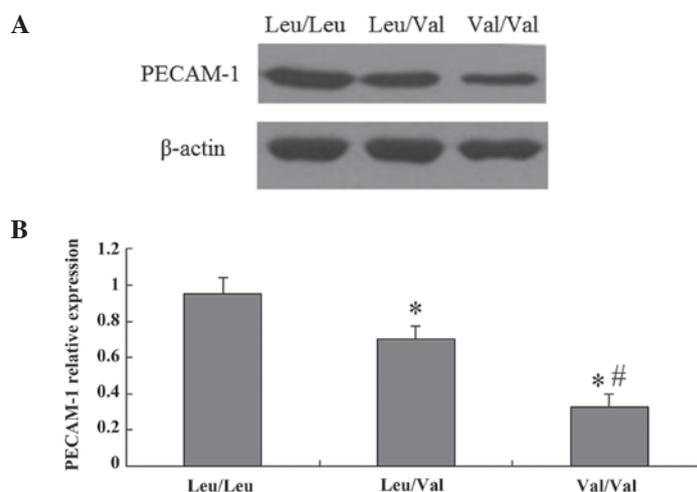


Figure 3. Lower protein expression levels of PECAM-1 were detected in the venous vessels of the DVT patients with Leu/Val and Val/Val genotypes, as compared with those possessing the Leu/Leu genotype. (A) Whole tissue extracts of venous vessels were immunoblotted with an antibody targeting human PECAM-1. Representative pictures from three independent experiments are shown. (B) Relative expression levels of PECAM-1 protein. The figure shows western blot results of DVT patients with Leu/Leu (n=6), Leu/Val (n=6) and Val/Val (n=6) genotypes. Relative intensity was calculated relative to the intensity of β -actin by densitometry. The Y axis indicates grey value of PECAM-1 normalized to that of β -actin. Data is presented as the mean \pm standard deviation. *P $<$ 0.05, as compared with the Leu/Leu genotype. #P $<$ 0.05, as compared with the Leu/Val genotype. DVT, deep vein thrombosis; PECAM-1, platelet endothelial cell adhesion molecule-1.

the Val/Val genotype as compared with those in patients with the Leu/Leu and Leu/Val genotypes. At 28 days post-diagnosis, the Δ thrombus scores for the patients with the Leu/Val and Val/Val genotypes were significantly lower

as compared with those for patients with the Leu/Leu genotype (Table V). These results indicated that the presence of the Val allele may promote delayed thrombus resolution in patients with DVT.

Discussion

The present study identified a significant association between DVT and the Leu125Val polymorphism of PECAM-1 and plasma sPECAM-1 levels. In patients with DVT, those with the Leu/Val and Val/Val genotypes exhibited increased levels of sPECAM-1 and decreased PECAM-1 protein expression levels in venous vessels. Furthermore, the Leu/Val and Val/Val genotypes were associated with an increased thrombus burden and delayed thrombus resolution in patients with DVT. However, associations were not observed between DVT and PECAM-1 Asn563Ser or Gly670Arg genotypes. These data suggested that PECAM-1 may have an important role in the development and thrombus resolution of DVT, and the Leu125Val polymorphism of PECAM-1 may serve as a novel genetic marker of susceptibility to DVT.

Virchow's triad, which includes abnormal blood composition and vessel wall components, and decreased blood flow, has been proposed to explain the pathophysiological mechanisms underlying the development of venous thrombosis (21). Out of the three components of the triad, blood composition, including circulating blood cells and plasma proteins, has been well studied, whereas the underlying mechanisms associated with the vessel wall and blood flow remain elusive. The early phase of venous thrombosis involves the recruitment of circulating leukocytes and platelets by adhesion molecules to sites of vascular damage. Platelet adhesion and enhanced procoagulant activity on endothelial cells is then modulated by shear stress (21). Therefore, adhesion molecules are considered to have a critical role in the process of venous thrombosis, which is supported by an increased risk of venous thrombosis associated with SNPs in the cell adhesion molecule 1 gene (22). PECAM-1 is another cell adhesion molecule, which is constitutively expressed in leucocytes, platelets and endothelial cells, and is involved in endothelial integrity and platelet function. A previous study suggested that PECAM-1 may be important in the inhibition of the adhesion cascade that leads to platelet activation and aggregation during the venous thrombosis process (23).

The present study identified an association between Leu125Val polymorphisms in PECAM-1 and DVT, with a significant increase in Leu/Val and Val/Val genotype frequencies and the Val allele in patients with DVT, as compared with those in control subjects. These results indicated a significantly increased risk of DVT in subjects with Leu/Val and Val/Val genotypes as compared with those possessing the Leu/Leu genotype. To the best of our knowledge, the present study was the first to examine PECAM-1 polymorphisms in patients with DVT. PECAM-1 SNPs have been widely reported to be associated with atherosclerosis and myocardial infarction (17,18), and previous studies have mainly focused on Leu125Val, Asn563Ser and Gly670Arg polymorphisms. However, in the present study, no significant differences were observed in the genotype and allele frequencies of Asn563Ser and Gly670Arg polymorphisms between the control and DVT groups. This may be due to the small number of study subjects and disparities between DVT and arterial diseases, including atherosclerosis and myocardial infarction.

Significantly higher plasma sPECAM-1 levels were detected in patients with DVT, as compared with the control subjects. In patients with DVT, sPECAM-1 levels were

significantly higher in those with the Val/Val and Leu/Val genotypes, as compared with levels in those with the Leu/Leu genotype. Plasma sPECAM-1 levels were previously shown to be elevated in patients with coronary artery disease (CAD), and patients with the Val/Val genotype of the Leu125Val polymorphism had higher sPECAM-1 levels (24). sPECAM-1 is generated either by alternative splicing upon cell activation, or PECAM-1 proteolytic cleavage at the cell surface (25,26). Leu125Val of PECAM-1 is located at exon 3, which encodes the first extracellular immunoglobulin (Ig)-like domain that mediates the homophilic binding of PECAM-1. These results indicated that Leu125Val is a functional SNP site and 125Val may enhance the production of sPECAM-1 in DVT.

The present study demonstrated that subjects with Leu/Val and Val/Val genotypes had an increased risk of DVT and higher sPECAM-1 levels, which contradict the previously observed inhibitory effects of PECAM-1 on thrombosis (27). Whether sPECAM-1 acts as an anti-thrombotic protein or only as a marker for DVT requires further study. The present study aimed to detect the protein expression levels of PECAM-1 in the venous vessels of patients with DVT, and PECAM-1 protein expression levels were shown to be significantly decreased in patients with Leu/Val and Val/Val genotypes, as compared with levels in those with the Leu/Leu genotype. Decreased PECAM-1 protein expression levels were previously detected in the venous vessels of patients with DVT with delayed thrombus resolution as compared with those in patients with normal thrombus resolution (14). The results of the present study further demonstrated that the Leu/Val and Val/Val genotypes of the Leu125Val polymorphism may decrease PECAM-1 protein expression in the venous vessels of patients with DVT. Previous studies have shown that PECAM-1 protein can be cleaved at the cell surface (28) and may exert competitive inhibition on membrane-bound PECAM-1 (29). Therefore, elevated sPECAM-1 plasma levels are most likely due to cleavage of PECAM-1 at the cell surface, which may lead to decreased PECAM-1 protein expression in the venous vessels of subjects with Leu/Val and Val/Val genotypes. These results indicated that plasma sPECAM-1 levels may be a marker for decreased PECAM-1 protein expression in venous vessels, and be associated with a high risk of thrombosis. This hypothesis was supported by a previous study that detected increased levels of plasma sPECAM-1 in polycystic ovary syndrome (30), which is a predisposing condition for VTE (31).

PECAM-1 has previously been shown to inhibit thrombus formation and PECAM-1-deficient mice exhibited larger thrombi as compared with those of control mice (32). To investigate whether decreased venous PECAM-1 protein expression due to the presence of the 125Val allele is associated with thrombosis in DVT patients, the thrombus burden of the patients was evaluated by complete compression ultrasonography. Patients with the 125Val allele had a significantly higher baseline thrombus score and lower Δ thrombus score, as compared with the 125Leu allele, thus indicating that the 125Val allele may promote thrombosis and delay thrombus resolution in patients with DVT. These results were concordant with the findings of a previous study that identified an association between delayed thrombus resolution and increased plasma sPECAM-1 levels, and decreased venous PECAM-1 protein expression in patients with DVT (14). The 125Leu allele of PECAM-1

gene was also shown to promote the thrombotic process and was associated with larger thrombi. The Leu125Val allele of PECAM-1 is located in the first extracellular (Ig)-like domain that mediates the homophilic binding of PECAM-1, and has an important role in maintaining the integrity of endothelial cell junctions (33). Further investigation is required to determine whether decreased thrombosis by 125Val is caused by decreased PECAM-1 expression, or defects in homophilic binding of the PECAM-1 protein. Furthermore, delayed thrombus resolution has a higher risk in developing into post-thrombotic syndrome (PTS) (34). In the present study, patients possessing the 125Val allele had a lower Δ thrombus score and delayed thrombus resolution. If these findings can be replicated by larger population studies, Leu125Val genotyping at DVT diagnosis may be used as a predictive marker to determine which patients are prone to PTS and require more aggressive treatment.

In conclusion, the present study demonstrated that subjects with the 125Val allele of PECAM-1 had an increased risk of DVT, increased plasma sPECAM-1 levels, decreased venous PECAM-1 protein expression, increased thrombus burden and delayed thrombus resolution. In DVT patients with the 125Val allele, higher plasma sPECAM-1 levels may be due to enhanced cleavage of PECAM-1 protein at the cell surface. The present study provides evidence suggesting that the Leu125Val polymorphism of PECAM-1 may be a potential genetic marker to predict susceptibility to DVT and PTS. Additional studies are required with larger sample sizes to confirm these findings. Further studies should focus on determining the pathways through which Leu125Val polymorphisms affect the anti-thrombotic effects of PECAM-1 in DVT.

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