Arteriosclerosis, Thrombosis, and Vascular Biology
JOURNAL OF THE AMERICAN HEART ASSOCIATION

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Arterioscler. Thromb. Vasc. Biol. 1999;19;1573-1576
Arteriosclerosis, Thrombosis, and Vascular Biology is published by the American Heart Association. 7272 Greenville Avenue, Dallas, TX 72514
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Complex Association of Protein C Gene Promoter Polymorphism With Circulating Protein C Levels and Thrombotic Risk

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Abstract**—**The allele and haplotype frequency of the –1654 *C/T* and –1641 *A/G* protein C (PC) gene promoter polymorphisms was determined and analyzed according to circulating PC concentrations in 394 healthy subjects aged 20 to 60 years. The *CG* haplotype was associated with a lower PC concentration in both homozygous and heterozygous subjects compared with noncarriers. The *TA* allele had the reverse effect, but only in homozygotes. The distribution of the *CG* and *TA* alleles was significantly different in 242 patients, aged 17 to 60 years, with venous thromboembolism. The *CG* allele increased the risk of thrombosis, with an OR of 1.39 (95% confidence interval (CI), 1.04 to 1.87). The *TA* allele was protective in subjects aged <45 years, with an OR of 0.68 (95% CI, 0.44 to 1.04). *TA* was also significantly associated with older age at the first thrombosis. This study confirms the link between the PC gene promoter and circulating PC levels, and suggests a complex effect on the risk of thrombosis. **(*Arterioscler Thromb Vasc Biol*. 1999;19:1573-1576.)**

Key Words: thrombosis ■ protein C ■ promoter ■ genetic risk

The protein C (PC)–protein S (PS) system is one of the most important mechanisms downregulating blood coagulation. PC is a vitamin K–dependent zymogen activated by the thrombin–thrombomodulin complex at the surface of endothelial cells. Activated PC forms a complex with its cofactor PS, and proteolytically inactivates activated factor V and factor VIIIa.[1]

Hereditary PC deficiency was first identified in subjects who had about half of the normal PC concentration and a family history of thrombosis.[2] In many families, a PC gene abnormality cosegregates with PC deficiency and with a thrombotic phenotype.[3] The thrombotic risk associated with low PC concentrations was confirmed in the Leiden thrombophilia case-control study of unselected patients with deep–venous thrombosis (DVT) before the age of 70: the relative risk increased as the PC concentration fell, with an odds ratio (OR) of ≈4 in individuals with PC levels <65%.[4] However, heterozygous subjects with a PC gene abnormality, and their noncarrier siblings, have overlapping PC concentrations, which suggests that other genetic factors may influence circulating PC levels.[5,6]

The human PC gene maps to chromosome 2q13-q14,[7] spans over 11 kb, and comprises a coding region (exons II to IX) and a 5′ untranslated region encompassing exon I.[8,9] Three polymorphic sites (–1654 *C/T*, –1641 *A/G*, and –1476 *A/T*) are located in the 5′ nontranscribed region of the PC gene. In 44 unrelated individuals in whom familial studies could be performed, 3 of 8 possible haplotypes (*CGT*, *TAA*, and *CAA*) were observed with frequencies of 0.33, 0.30, and 0.25, respectively.[10] Interestingly, in a population of 240 patients and controls, subjects homozygous for the *CGT* combination had lower PC concentrations than subjects homozygous for the *TAA* combination.[11] Each polymorphic site was analyzed in the 474 patients and controls of the Leiden Thrombophilia Study, and individuals with the homozygous *CGT* genotype were found to have a 50% to 100% greater risk of venous thrombosis than individuals with the homozygous *TAA* genotype.[11] The limitation of this study was that only *CGT* and *TAA* homozygotes were precisely genotyped.

The present study was designed to determine the genotype and allele frequencies of these polymorphisms in a large population, to identify a link with the PC concentration in normal subjects, and, if the effect of the polymorphisms on PC gene expression was confirmed, to seek a link with the risk of thrombosis. According to Scope,[12] the transcriptional efficiency of these haplotypes is driven by the polymorphisms at positions –1654 and –1641.[12] We thus used a method that allowed the *C/T* and *A/G* haplotype (theoretically *CG*, *CA*, *TA*, and *TG*) to be determined on an amplified fragment encompassing both the –1654 and –1641 polymorphic sites. The frequency of the PC promoter haplotypes

Received March 9, 1998; revision accepted November 17, 1998.

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and their different combinations were determined in 394 control subjects and 242 patients with DVT.

Methods

Subjects

Healthy subjects, aged 20 to 60 years, were recruited between May and September 1996 from a health care center to which they had been referred for a routine checkup. None of these subjects had a history of arterial disease (stroke, myocardial infarction, angina, or peripheral vascular disease), venous thrombosis (pulmonary embolism or DVT), or known malignancy, as reported on a medical questionnaire.

Patients with venous thromboembolism (VTE) were recruited from the vascular medicine unit of a Paris hospital from November 1995 to June 1998 and were enrolled onto the study if they were younger than 61 and had experienced at least 1 episode of objectively diagnosed DVT (compression ultrasonography or venography) and/or pulmonary embolism (perfusion and ventilation lung scan, conventional pulmonary angiography, or computed tomographic angiography). Blood sampling for DNA analysis, demographic data, and clinical characteristics recording were performed at the time of inclusion. The study was approved by the local ethics committee and all the subjects gave their informed consent.

Laboratory Investigations

Venous blood was collected onto 0.129 M trisodium citrate (1:10) and plasma was kept frozen until use.

PCantigen (PCag) was assayed by an immunoenzymatic method (Asserachrom Protein C, Diagnostica Stago). Control plasma was assayed within 3 months using a pool of plasma from 50 healthy men, aged 20 to 60, to construct the reference curve.

Molecular Biology

The PC gene promoter was analyzed after polymerase chain reaction amplification of the fragment of the promoter encompassing nucleotides –1738 to –1596 in the numbering system of Foster et al.[9] Denaturing gradient gel electrophoresis (DGGE) of the amplified fragment was performed as described elsewhere.[13] Using this method, the haplotypes (*CG*, *CA*, *TA*, and *TG*) resulting from combinations of the 2 polymorphic sites (–1654 and –1641) can easily be determined. However, we never encountered the *TG* haplotype in a genotyping study of >1000 subjects.

The factor V Arg506Gln mutation was identified using a previously described method.[14]

Statistical Analysis

Data were analyzed using the SAS statistical software (SAS Institute Inc). Hardy-Weinberg equilibrium was tested by a χ^2 test with 2 degrees of freedom (*df*) separately in cases and controls. Allele frequencies were deduced from the genotype frequency.

The level of PCag was studied in controls according to genotypes. The *CG* and *TA* genotypes were each studied as a biallelic polymorphism by reference to *CA-CA* carriers: homozygotes (*CG-CG* or *TA-TA*), heterozygotes (*CG*+ or *TA*+), and noncarriers. Both *CG* and *TA* genotypes (2 *df* each) were entered into a linear model (PROC GLM) with the dependent variable being PCag, adjusting for age and sex.

The ORs for DVT associated with 1 copy of *CG* and *TA* were calculated by a logistic regression procedure (SAS-PROC LOGIST), where both genotypes were entered together. *CG* and *TA* were coded as 2-ordered variables with 1 *df*, assuming additive effects of alleles (ie, tested as continuous): 0, noncarriers; 1, heterozygotes for *CG* or *TA*, respectively; 2, homozygotes for *CG* or *TA*, respectively. Thus the ORs are for 1 copy of the *CG* or *TA* allele. The log-likelihood of this model was not significantly different from that of a model where the genotypes were coded as 2 dummy-variables, contrasting the effect of heterozygotes and homozygotes to noncarriers (equivalent to single variables with 3 unordered categories, assuming no particular genetic model).

The recurrence of thrombosis, associated pulmonary embolism, and VTE associated with acquired risk factors (pooling contraception, pregnancy, surgery, prolonged immobilization, or cancer) according to genotype was tested in cases by a χ^2 test.

The difference in age at first thrombosis was tested by a general linear model adjusted for sex, where both *CG* and *TA* genotypes were entered.

The homogeneity of the results in men and women, and across age, was systematically tested by entering the corresponding interaction term. $P<0.05$ was considered significant.

TABLE 1. Characteristics of Cases With Deep-Venous Thrombosis (DVT) and Controls

	Cases n=242	Controls n=394	Test *P*
% Women	55.8 (3.2)	49.5 (2.5)	0.12
Mean age	42.2 (11.3)	43.0 (9.5)	0.34
% Oral contraception in women	33.3 (4.1)	19.0 (2.8)	0.003
% FV-Q506 mutation	19.5 (2.8)	3.8 (1.0)	<0.001
% DVT associated with acquired risk factors*	59.5 (3.2)	...	...
% Recurrent DVT	27.5 (2.9)	...	...
% Pulmonary embolism	40.1 (3.2)	...	...
Mean age at first DVT	37.7 (12.2)	...	...

SD in parentheses.

*Contraception, pregnancy, surgery, prolonged immobilization, or cancer.

Results

Table 1 shows the characteristics of the study population. Cases and controls were well-matched in terms of sex and age. As expected, the factor V Arg506Gln mutation was observed in 19.5% of cases and 3.8% of controls ($P<0.001$). Women on oral contraception (a known risk factor for DVT) were more frequent among cases than among controls. The occurrence of thrombosis was associated with a known acquired risk factor in 59.5% of the cases. VTE was recurrent in 27.5% of the cases, and 40.1% of the cases had pulmonary embolism.

The distribution of the PC gene promoter *CG*/*TA*/*CA* genotypes in the 394 controls is shown in Table 2. There was no significant deviation from Hardy-Weinberg equilibrium in the controls. *CG* was the most frequent allele and *CA* the least frequent.

TABLE 2. Distribution of the Poly-*CG*/*TA*/*CA* Genotypes and Allele Frequencies in Cases With DVT and Controls

	Cases		Controls	
	n	%	n	%
CD-CG	56	23.2	69	17.5
CG-TA	76	31.4	107	27.2
CG-CA	46	19.0	69	17.5
TA-TA	21	8.7	52	13.2
TA-CA	33	13.6	75	19.0
CA-CA	10	4.1	22	5.6
Total	242	100.0	394	100.0
CG allele frequency	0.483		0.398	
TA allele frequency	0.312		0.363	
CA allele frequency	0.205		0.239	
Difference in allele distribution between cases and controls	$P=0.012$			

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TABLE 3. Mean Protein C Antigen Level According to *CG* and *TA* Genotypes in Controls

	CG Genotype		*TA* Genotype	
	n	Mean (SEM)	n	Mean (SEM)
Noncarriers	149	111.5 (1.4)	160	104.5 (1.4)
Heterozygotes	176	106.0 (1.6)	182	104.4 (1.5)
Homozygotes	69	103.1 (2.5)	52	111.9 (2.7)
		P=0.0095		P=0.03

Means were adjusted for age and sex. These effects were not significantly heterogeneous across sex or age.

The respective influence of the *CG*, *TA*, and *CA* genotypes on the PC plasma concentration (immunoassay) was then analyzed in the controls. As shown in Table 3, *CG* was associated with significantly lower PC concentrations, with mean values of 103.1%, 106.0%, and 111.5% in homozygotes, heterozygotes, and noncarriers, respectively, after adjustment for age and sex ($P<0.01$). Conversely, the PC concentration was elevated in *TA* homozygotes (111.9%; $P<0.05$), relative to heterozygous (104.4%) and noncarriers (104.5%). These effects were not significantly heterogeneous according to sex or age. The presence or absence of *CA* did not modify the PC concentration. These results confirm and extend a previous observation that the PC concentration is genetically determined in healthy individuals.[11]

Because the thrombotic risk correlates negatively with the PC concentration,[4] we genotyped the 242 patients with VTE for the PC promoter polymorphisms (Table 2). There was no significant deviation from Hardy-Weinberg equilibrium in the cases. The *CG* frequency was higher in the cases than in the controls, and the *TA* frequency was higher in the controls than in the cases (P=0.012), inferring a significant effect of the *CG* and *TA* genotypes on the risk of DVT. The OR associated with 1 copy of *CG* was 1.39 (95% CI, 1.04 to 1.87; $P<0.028$), with no effect of age or sex, confirming that *CG* significantly increased the risk for thrombosis in this population. As shown in Table 4, although *TA* had no effect when subjects of all ages were included in the analysis, with an OR of 1 (95% CI, 0.72 to 1.37; P=0.98) for 1 copy of *TA*, the effect was significantly influenced by age ($P<0.01$): the *TA* allele tended to reduce the risk in subjects under 45 (median age), with an OR of 0.68 (95% CI, 0.44 to 0.1.06; P=0.09), whereas it tended to increase the risk in subjects over 45, with an OR at 1.56 (95% CI, 0.96 to 2.51; P=0.07).

TABLE 4. Odds Ratios (95% CI) for Deep–Vein Thrombosis Associated With *CG* and *TA* Alleles, all Ages

	CG Genotype		*TA* Genotype	
	n Cases	n Controls	n Cases	n Controls
Noncarriers	64	149	112	160
Heterozygotes	122	176	109	182
Homozygotes	56	69	21	52
OR (95% CI)	1.39 (1.04 to 1.87)		1.00 (0.72 to 1.37)	
	P=0.028		P=0.98	

Heterogeneity of the ORs associated with one copy of *CG* across age: NS. Heterogeneity of the ORs associated with one copy of *TA* across age: $P<0.01$. The effects were not significantly influenced by sex.

The clinical circumstances of the thromboses were then analyzed according to the PC promoter genotype. We observed an effect of the *TA* allele on age at the first thrombosis, which occurred at a mean (adjusted for sex) of 36.4 years in noncarriers, 38.8 years in heterozygotes and 44.2 years in homozygotes (P=0.07; assuming an additive effect of alleles, P=0.03). The *CG* allele had no such effect on age at onset.

The presence of acquired risk factors for thrombosis was also analyzed in *CG* and *TA* carriers. The *CG* allele was not significantly related to the type of thrombosis (spontaneous or acquired). In the younger patients (<45), acquired risk factors were less frequent among *TA* carriers: 57% in carriers versus 79% in noncarriers (P=0.008, 1 *df*). This effect, suggesting a protective effect of *TA* on development of thrombosis associated with acquired risk factors, was not observed in patients >45 years old.

No link was found between PC gene promoter polymorphism and the recurrence of thrombosis.

Discussion

We took advantage of the proximity of 2 frequent polymorphisms of the PC gene promoter to determine haplotypes with the DGGE technique in 636 subjects from Paris. Three hundred ninety-four of these subjects, aged 20 to 60 years, were recruited in a health center if they reported no history of venous or arterial thrombosis. Two hundred forty-two patients, aged 17 to 60 years, with confirmed VTE were recruited in a hospital vascular medicine unit. The controls and cases all lived in the Paris area, but there were no geographic or ethnic criteria for eligibility. It was thus important to check that the 2 populations were comparable with other European populations in terms of genetic risk factors for thrombosis. The frequency of the factor V mutation was 19.5% in the patients with thrombosis (cases) and 3.8% in the subjects without thrombosis (controls). These frequencies are similar to those observed in other studies.[15,16] The distribution of the prothrombin 20210A allele (10.2% versus 2.8% in cases and controls, respectively), which has been found to increase the risk of venous thrombosis,[17] was also similar to that seen in other European populations.[18]

The respective frequencies of the *CG*, *TA*, and *CA* alleles were 39.8%, 36.3%, and 23.9% in our 394 French controls; 35.3%, 28.4%, and 32.4% in 102 British subjects[12]; and 36.4%, 30.7%, and 28.4% in 88 Dutch subjects.[10] The *CG* and *TA* alleles were thus slightly more frequent in our population, whereas the *CA* allele was less frequent. It is noteworthy that we never found the *TG* haplotype in the 636 subjects tested, whereas 3.9% and 4.5% of British and Dutch subjects, respectively, had this haplotype.[10,12]

The PC concentration was measured in the 394 controls using an immunoenzymatic assay, and the results confirmed the effect of the *CG* and *TA* alleles on PC gene expression. This effect had initially been established by comparing 40 individuals homozygous for the *CG* genotype to 28 individuals homozygous for the *TA* genotype.[11] The results presented here show that not only homozygous individuals, but also heterozygous individuals, for the *CG* haplotype have lower PC concentrations than individuals who do not carry *CG*. Conversely, the *TA* haplotype increased the PC concentration, but only in homozygotes. As the risk of venous thrombosis correlates negatively with the PC concentration,[4] a detrimen-

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tal effect of the *CG* allele could be expected. The *CG* allelic frequency was indeed significantly higher in patients with DVT than in controls (48.3% versus 39.8%). This indicates that individuals with the *CG* allele (1 copy, more so for 2 copies) have a higher risk of thrombosis than noncarriers. The risk of thrombosis was significantly increased (1.39-fold; 95% CI, 1.04 to 1.87), and the effect was homogeneous across age and sex.

The effect of the *TA* allele was more complex, being dependent on age. In patients under 45 (median age), the OR for thrombosis associated with 1 *TA* allele was 0.68 (95% CI, 0.44 to 1.06). This potentially protective effect was not observed after 45 years; on the contrary, the *TA* allele tended to increase the risk in this subgroup, with an OR of 1.56 (95% CI, 0.96 to 2.51).

A protective effect of *TA* is consistent with the finding of higher PC levels in *TA* control subjects. We cannot exclude a bias accounting for the fact that the *TA* protective effect was limited to the younger patients. There might be confounding factors in the older patients explaining the heterogeneity of the results, but the study of such factors requires a larger series of patients.

Also consistent with the protective effect of *TA* in younger adults is the observed link between *TA* and age at the first thrombosis. Thrombosis occurred later in *TA* carriers, with mean ages of 36.9, 39.5, and 44.6 years in noncarriers, heterozygotes, and homozygotes, respectively. No such effect was observed with the *CG* allele.

The last finding in this study was the link between the *TA* allele and known acquired risk factors for thrombosis. In the Leiden Thrombophilia Study, the distribution of acquired risk factors was not influenced by PC levels.[4] It remains to be determined why the *TA* allele, which seemed to have a protective effect in subjects under 45 in our study, was more frequent in patients with spontaneous thrombosis than in those with circumstantial risk factors. The observation that *TA* mainly reduces the risk of thrombosis in patients under 45 with acquired risk factors suggests that it interacts with risk factors specific to younger people, such as trauma, oral contraception, and pregnancy. The patients with the *TA* allele and thrombosis might thus have unknown genetic abnormalities overwhelming the apparent protective effect of the *TA* allele.

The study of a large population of healthy subjects allowed us to confirm the link between the *CG* allele and lower PC concentrations, and to show the opposite effect of the *TA* allele in homozygotes. Carrying 1 copy of the *CG* allele increased the risk of thrombosis by 4% to 87% whereas the *TA* allele had a protective effect in subjects under 45. This illustrates the complexity of the genetic factors involved in the risk of venous thrombosis.

Acknowledgments

This work was supported by a grant from *Programme Hospitalier de Recherche Clinique* number AO94031 "*Evaluation clinique et biologique du risque thrombotique*". We thank Marie-Laurence Aubry for her excellent technical assistance and José Bon-Deguingand for her excellent secretarial assistance.

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